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13. ABSTRACT (Maximum 200 Words) A high incidence of familial breast and ovarian cancer is associated with inactivation of BRCA1. We demonstrated previously that HRG induced the phosphorylation of BRCA1, which was mediated by the phosphatidylinositol-3 kinase (PI3K)/Akt pathway. Since both extracellular matrix (ECM) and PTEN can modulate PI3K/Akt pathway we hypothesized that ECM and PTEN may affect the expression and phosphorylation of BRCA1. To test this we wanted: 1) To determine the effect of ECM/integrins on BRCA1 expression, phosphorylation and nuclear translocation. 2) To characterize biological functions of BRCA1 in breast cancer cells. 3) To determine the effect of PTEN on BRCA1 phosphorylation. Both cell proliferation and BRCA1 phosphorylation were enhanced in T47D cells seeded on laminin after treatment with heregulin (HRG). The enhanced BRCA1 phosphorylation on laminin was mediated through $\alpha_6\beta_4$ integrins. Overexpression of BRCA1 inhibited HRG-dependent DNA synthesis in breast cancer cells. HRG suppressed BRCA1 expression through protein degradation, which required both calpain and proteasome. ECM suppressed BRCA1 mRNA expression through its C-terminus, while forced expression of PTEN inhibited HRG-dependent activation of Akt. Taken together these findings show that while BRCA1 suppresses HRG-dependent DNA synthesis, ECM and/or HRG can regulate BRCA1 expression and phosphorylation. Experiments with PTEN indicated that this phosphatase could affect phosphorylation of BRCA1.				
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## **INTRODUCTION**

BRCA1 is tumor suppressor gene whose inactivation is associated with a high incidence of familial breast and ovarian cancer [1]. The BRCA1 protein can be phosphorylated by heregulin through phosphatidyl inositol-3 kinase (PI3K)/AKT-dependent pathway [2] and cell cycle-dependent manner [3]. The phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumor suppressor gene located at 10q23 [4]. Deletions in 10q22-25 occur in multiple tumor types and PTEN is one of the most common targets of mutations in human cancer [5, 6]. PTEN dephosphorylates PtdIns phosphates both *in vitro* and *in vivo* [7] and inhibits the PI3K-dependent activation of AKT [8]. The extracellular matrix (ECM) plays important role in morphogenesis, tissue repair and regeneration as well as metastasis of mammary glands [9-11]. ECM elicits its action through integrins, which have been shown to activate PI3K [12]. We hypothesize that (i) inhibition of normal BRCA1 function by heregulin could contribute to the dysregulated cell growth, (ii) BRCA1 expression and phosphorylation might be regulated by various signaling pathways. Since PTEN and ECM have been reported to regulate PI3K/AKT pathway, we aimed to characterize their role in regulating BRCA1 expression, phosphorylation and function. Based on these hypotheses, our specific aims are: 1) to determine the effect of ECM/integrins on BRCA1 expression, phosphorylation and nuclear translocation. 2) To generate inducible stable transfected T47D clones that over-express BRCA1 protein, in order to characterize its biological functions in breast cancer cells. 3) To determine the effect of PTEN on BRCA1 phosphorylation.

## **BODY**

### Extracellular matrix (ECM) modulates heregulin (HRG)-dependent mitogenic response of T47D cells.

T47D breast cancer cells proliferate uniformly when treated with growth factors [13]. To study the effect of substratum on the mitogenic response of T47D cells, the incorporation of [<sup>3</sup>H]thymidine into DNA was measured. Cells grown on plastic (PL) showed a maximal DNA synthesis between 12 and 20 h, peaking at 18 h after treatment with heregulin (HRG, 209  $\pm$  22% vs. 100  $\pm$  38% of untreated cells; Figure 1A). A similar response was obtained when T47D cells were grown on fibronectin (FN, 206  $\pm$  13%, at 18 h). Cells seeded on collagen type IV (COL4) strongly suppressed the mitogenic response (92  $\pm$  38%, at 18 h), whereas the peak of thymidine incorporation was markedly increased in those cells grown on laminin (LAM) or Matrigel (MTR) (276  $\pm$  10% and 264  $\pm$  11%, at 18 h, respectively). Cells seeded on poly-L-lysine (POL) showed no difference in DNA synthesis whether in the presence or absence of HRG (100  $\pm$  16% vs. 100  $\pm$  13%), suggesting the involvement of integrins, receptors for the extracellular matrix, in HRG-dependent mitogenic response. Indeed, T47D breast cancer cells were found to express  $\alpha_6$ ,  $\beta_1$  and  $\beta_4$  integrins, as indicated in Figure 1E. T47D cells adhered similarly to all substrata used in this study [PL, LAM, FN, COL4 and POL, (data not shown)] suggesting that the differences in mitogenic response were not due to different number of cells attaching to different substratum. Thus, T47D cells proliferated to a comparable level when seeded on PL and FN, while demonstrating decreased growth when seeded on collagen type IV or on poly-L-lysine. Cells grown on LAM or on MTR demonstrated the highest mitogenic response upon treatment with HRG.

To determine if ECM would affect DNA synthesis elicited by other growth factors, we compared the mitogenic response of cells stimulated with epithelial growth factor (EGF, 10 ng/ml, Figure 1C) or fetal bovine serum (FBS, 10%, Figure 1D) to that elicited by HRG (Figure 1B). A representative time point of thymidine incorporation (at its peak; 18 h) was compared to non-treated cells (basal level, taken as 100%). While cells seeded on LAM and MTR responded to HRG with enhanced DNA synthesis, cells seeded on PL and FN as well as those grown on MTR had the greatest response to EGF (367  $\pm$  11%, 290  $\pm$  13% and 262  $\pm$  4%, respectively vs. 100  $\pm$  20% of non-treated cells, Figure 1C). Although cells grown on PL (237  $\pm$  4%), LAM (320  $\pm$  8%) or MTR (259  $\pm$  9%) had a considerably high thymidine incorporation, those cells grown on FN had the highest peak of DNA synthesis after treatment with FBS (586  $\pm$  8%). Interestingly, DNA synthesis was strongly suppressed on both POL (118  $\pm$  10% at 18 h) and COL4 (97  $\pm$  33% at 18 h) coated plates after treatment with EGF, while cells treated with FBS responded modestly when seeded on these two matrices (POL, 152  $\pm$  45%; COL4, 171  $\pm$  39%, at 18 h). This modest effect could be attributed to the mixture of growth factors and cytokines found in FBS. Thus, laminin and also laminin-containing Matrigel had the strongest effect in enhancing HRG-dependent mitogenic response in T47D breast cancer cells.



### ECM modulates the HRG-dependent phosphorylation of BRCA1.

BRCA1 can be phosphorylated in response to DNA damage and in a cell cycle-dependent manner. Previously, we reported that BRCA1 was phosphorylated upon HRG stimulation [2]. To investigate whether ECM may modulate BRCA1 phosphorylation, cells were grown on plastic plates, on plates coated with either LAM or POL, or were maintained in suspension. Cells were starved for 48 h and metabolically labeled with [ $^{32}$ P]H $_3$ PO $_4$ . The phosphorylation of BRCA1 was then determined by immunoprecipitation with anti-BRCA1 antibody, followed by separation on SDS-PAGE. Cells seeded on PL showed a transient phosphorylation of BRCA1 in response to HRG, peaking at 0.5 – 1 h and returning to the basal level thereafter (Figure 2A), supporting our previous work [2]. Therefore, at the 1 h time point, representing the peak of HRG-dependent phosphorylation, the effect of ECM components on this process was tested. While the amount of BRCA1 protein was constant throughout the experiment as indicated in the lower panels of Figure 2, the level of phosphorylated BRCA1 upon HRG treatment was enhanced in cells seeded on LAM as compared to those seeded on PL (Figure 2B). The increased BRCA1 phosphorylation on LAM was suppressed by blocking antibodies for  $\alpha_6$  integrin, while blocking anti- $\beta_1$  integrin antibody had a modest effect, as indicated in Figure 2C. Cells seeded on COL4 or those maintained in suspension showed no or very low phosphorylation of BRCA1. Taken together, these results indicate that LAM enhanced the HRG-mediated phosphorylation of BRCA1. This phenomenon was mediated by  $\alpha_6$  integrin.

### Is HRG-dependent signaling enhanced in cells seeded on laminin?

HRG-dependent phosphorylation of BRCA1 is regulated through the PI3K/Akt pathway after recruitment of PI3K to the activated receptor [2, 14]. To investigate further the HRG-dependent intracellular signaling, cells were seeded on plastic, on plates coated with FN, COL, or POL, or were maintained in suspension and then compared to those grown on LAM. HRG increased the activity of various tyrosine kinases, which peaked at 10 min, as determined by anti-phosphotyrosine antibody (not shown). Therefore, the effect of ECM on HRG-dependent downstream signaling was tested at 10 min, the peak of tyrosine phosphorylation induced by HRG. Total cell lysates were immunoprecipitated with anti-ErbB-2 antibody, subjected to SDS-PAGE and probed with anti-phosphotyrosine antibodies (Figure 3A). Cells seeded on PL showed a strong increase in ErbB2 tyrosine phosphorylation. This phosphorylation was 2-3-fold stronger in cells grown on LAM, thereby corroborating the previous observations that LAM supported the strongest phosphorylation of BRCA1 and mitogenic response (Figure 2B and Figure 1B). Cells seeded on FN or on COL4 showed stimulation of the ErbB-2 receptor, however, it was much lower than that seen in cells grown on PL. The lowest tyrosine phosphorylation was observed in cells seeded on POL or in cells maintained in suspension (Figure 3A).

### Laminin increases HRG-dependent PI3 kinase activity but not MAPK activity.

PI3K activity was measured by an *in vitro* kinase assay and by western blotting using anti-phospho-Ser<sup>473</sup> AKT antibodies. The highest PI3K activity was observed in cells grown on LAM, and the lowest was seen on FN and COL4 (Figure 3B and C). Samples from untreated cells were not able to significantly phosphorylate phospholipids. To address the specificity, several kinase inhibitors were used prior to the addition of HRG. As expected, LY294002 totally inhibited HRG-dependent PI3K activity in these cells, while PD98059, a known MEK inhibitor, was not effective (Figure 3B and C). Similar to our previous study [2], MAPK did not play a significant role in this process as determined by western blotting using anti-phospho-MAPK antibodies and by an *in vitro* kinase assay (Figure 4A and B, respectively). The strongest MAPK activity was observed in cells grown on PL or FN, while cells grown on COL4 or LAM, or those maintained in suspension showed modest MAPK activity after treatment with HRG (Figure 4A and B). Therefore, the highest BRCA1 phosphorylation as seen in cells grown on LAM (Figure 2B) also correlated with greater intracellular signaling on the same substratum (Figure 3).

### Effect of ECM on BRCA1 expression

Because ECM affected the phosphorylation of BRCA1 in the presence of growth factors, we analyzed whether ECM also affects the expression of BRCA1. Over an extended time course, we observed a biphasic effect of HRG on BRCA1 mRNA levels in T47D cells grown on plastic. Specifically, we noted a high basal level of BRCA1 mRNA in non-treated cells which decreased rapidly up to 4 h after HRG treatment. This was followed by an increase in BRCA1 mRNA, peaking at 12 – 16 h, and by a decrease thereafter which approached the basal

level (not shown). Based on these initial observations, we decided to compare the effect of ECM components on the expression of BRCA1 in non-treated or HRG-treated cells. The five most characteristic times were taken as study points including 0, 4, 14, 24, and 48 h after treatment with HRG and then compared to those time points after treatment with DMSO (Figure 5A, B, and C). A characteristic biphasic pattern of BRCA1 expression after the treatment with HRG was observed in cells grown on plastic, laminin or collagen type IV. However, there was lower expression of BRCA1 in cells grown on laminin and the lowest expression in cells grown on collagen type IV (Figure 5A, B, and C). This decrease of BRCA1 mRNA in T47D cells after growth on either laminin or collagen type IV was not observed in HCC-1937 breast cancer cells (Figure 5D). This result suggests that this phenomenon was mediated through the C-terminus of the BRCA1 molecule, as HCC-1937 cells express C-terminus truncated BRCA1 [15]. In order to test whether this strong decrease in BRCA1 mRNA expression upon HRG treatment is dependent on protein synthesis or the stability of the mRNA, cells were treated with actinomycin-D or with cycloheximide. There was no difference in BRCA1 mRNA pattern when cells were treated with HRG alone (Figure 6A) or HRG together with actinomycin-D (Figure 6B). However, treatment of cells with cycloheximide (Figure 6C) reversed the effect of HRG, as the mRNA expression of BRCA1 was increased 2 and 4 h after addition of the growth factor. This suggests the dependence of this phenomenon on protein synthesis. To further elucidate the decrease in BRCA1 mRNA level 4 h after treatment with HRG, cells were also treated with ALLN (a calpain inhibitor) or with lactacystin (a proteasome inhibitor) prior to the addition of HRG. As indicated in Figure 6D, the HRG-dependent decrease in the BRCA1 mRNA level was not affected by pretreatment of cells with ALLN or lactacystin alone, while both inhibitors combined overcame the effect of HRG. In addition, another proteasome inhibitor, PSI, had a modest effect on the HRG-dependent BRCA1 mRNA level when compared to that of ALLN and lactacystin together (not shown).

#### Effect of ECM and HRG on BRCA1 protein levels in T47D cells

The strong basal level (Figure 7A) of BRCA1 protein was decreased 4 h after treatment with HRG (Figure 7B and 8A). Treatment of cells with DMSO, a solvent for HRG, did not decrease the level of BRCA1 protein (Figure 7A and B). Cells were also seeded onto collagen type IV and laminin to test whether these matrices would affect protein expression as they did the BRCA1 mRNA. Doublet bands of BRCA1 protein were detected in the total lysates from cells seeded on both laminin and collagen type IV, which could be attributed to alternative splicing of the BRCA1 protein. Cells grown on collagen IV or on laminin had comparable levels of BRCA1 protein in non-treated cells (Figure 7C and D). Similar to the cells adhering to plastic, there was a decrease in BRCA1 protein level 4h after stimulation with HRG, and this level further decreased up to 24 h (Figure 7C and D). The protein loading did not affect the results as the membranes were stripped and re-probed with either anti-actin antibody or with anti- ErbB2 antibody (Figure 7A, B, C and D, lower panels). Similar to the expression of mRNA, the low BRCA1 protein level 4 h after treatment with HRG was restored after pre treatment with the proteasome inhibitor, PSI, suggesting the involvement of proteasome in this process (Figure 8A). Four-hour exposure of T47D cells to cycloheximide or to actinomycin-D, together with HRG, down-regulated BRCA1 protein to undetectable levels, a similar effect as when cells were treated with HRG alone. The results also suggest the involvement of both processes, protein synthesis and the stability of mRNA, in the suppressive effect of HRG on BRCA1 protein expression in these cells.

#### The effect of PTEN on BRCA1 phosphorylation

Over-expression of PTEN in 293T cells confirmed that we had a right construct as detected by both anti-PTEN and/or anti-HA antibodies (shown in last annual report). Because 293T cells do not express ErbB2 receptors and because T47D cells have very low efficiency of transfection we acquired MDA-MB-453 cells that over-express ErbB2 and that have higher transfection efficiency than the T47D cells to test the effect of PTEN on HRG-dependent phosphorylation of BRCA1. MDA-MB-453 cells treated with HRG showed a transient phosphorylation of Akt peaking at 5 min upon addition of the agonist (Fig. 9) that was inhibited by forced expression of wild type (WT)-PTEN. Inactivating point mutation (cysteine replaced by serine, C/S) reversed the inhibitory effect of PTEN, while a non-meaningful point mutation (glycine replaced with arginine, G/R) was without effect (Fig. 9). This suggests that over-expression of PTEN would also inhibit the HRG-dependent BRCA1 phosphorylation which we will test in next stage of experiment.

## **KEY RESEARCH ACCOMPLISHMENTS**

1. ECM affected (laminin enhanced, collagen IV suppressed) DNA synthesis in breast cancer cells.
2. ECM (laminin) enhanced HRG-dependent phosphorylation of BRCA1 through  $\alpha_6\beta_4$  integrins.
3. HRG-dependent signaling leading to BRCA1 phosphorylation is enhanced on laminin.
4. HRG suppresses mRNA and protein expression of BRCA1.
5. ECM (laminin and collagen IV) suppressed BRCA1 mRNA expression through its C-terminus.
6. PTEN inhibits activation of Akt in breast cancer cells.
7. BRCA1 suppresses HRG-induced DNA synthesis in T47D cells.

## **REPORTABLE OUTCOMES**

1. Poster presentation at 92. Annual Meeting of American Association for Cancer Research, San Francisco, April 6-10, 2002, Poster # 5182 "Extracellular matrix effects the heregulin-dependent phosphorylation and expression of BRCA1 in breast cancer cells". (see Appendix B)
2. A manuscript submitted to Journal of Molecular and Cellular Biology under title: "Extracellular matrix enhances heregulin-dependent BRCA1 phosphorylation and suppresses BRCA1 expression through its C-terminus in breast cancer cells. (see Appendix C)

## **CONCLUSIONS**

Both HRG and ECM affected the expression and phosphorylation of BRCA1 in breast cancer cells. While HRG induced BRCA1 phosphorylation through PI3K/Akt-dependent pathway (Fig. 2), it also induced a strong decrease in BRCA1 mRNA (Fig. 5) and protein (Fig. 7 and 8) level through protein degradation. Laminin enhanced HRG-dependent BRCA1 phosphorylation through  $\alpha_6\beta_4$  integrins (Fig. 2) which resulted in increased cell proliferation (Fig. 1), whereas both laminin and collagen type IV caused a C-terminus-dependent decrease in BRCA1 mRNA expression (Fig. 5). However, collagen type IV suppressed the HRG-dependent mitogenic response in these cells (Fig. 1). Over-expression of wild type of PTEN inhibited HRG-dependent activation of Akt (Fig. 9).

In an early stage of a breast cancer an intact basement membrane, containing laminin and collagen type IV, surrounds cancerous cells and thereby regulates the expression and phosphorylation of BRCA1. Wild type BRCA1 would suppress proliferation and tumorigenicity of these cells. Interactions of breast cancer cells with laminin will cause hyper-phosphorylation of this tumor suppressor gene and cause decrease in its expression, which could result in increased cell proliferation and enhanced overall tumorigenicity. Interaction of breast cancer cells with collagen type IV, although, it may cause down-regulation of BRCA1 mRNA level, inhibits proliferation of breast cancer cells. A gradual mutation of BRCA1 and/or matrix degradation will eventually lead to development of metastatic phenotype of breast cancer cells.

During the course of the present studies Dr. Miralem learned several new laboratory techniques such as lipid phosphorylation assay (necessary for PI3K activity), confocal microscopy (for intracellular localization of proteins), some aspects of molecular biology including insert ligation, forced protein expression. He also presented his work at several symposia and seminars, such as AACR meeting (twice, poster), Beth Israel Deaconess Medical Center annual meeting (poster session) and our departmental seminars (Experimental Medicine).

## **FUTURE PLANS**

1. Characterize the effect of PTEN on BRCA1 phosphorylation.
2. Characterize intracellular localization of BRCA1; effect of HRG, ECM and phosphorylation.
3. Examine the mechanism of ECM-dependent inhibition of BRCA1 expression.
4. Further elucidate the mechanism by which HRG down-regulates BRCA1 expression.

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## **FIGURE LEGENDS**

### **FIG. 1. Effect of ECM on the HRG-dependent mitogenic response of T47D cells.**

(a) T47D cells were seeded on either 24-well plastic plates ( PL; ● , ○ ) or on plastic wells coated with fibronectin (FN, ▲), laminin (LAM, □ ), matrigel (MTR, ■ ), collagen type IV (COL4, ◇ ) or poly-L-lysine (POL, △ ) and then starved for 48 h in medium containing 0.4% FBS. Cells were untreated ( ○ ) or treated with HRG (20 nM) for the indicated times and the medium was replaced with one that contained [<sup>3</sup>H]thymidine (2 μCi/ml). After 45 min, the “hot” medium was washed out, cells were lysed with NaOH and cell remnants were collected into scintillation tubes to measure incorporated radioactivity.

To compare the effect of HRG (a and b) with other growth factors, cells seeded as described in (a) were treated with EGF (10 ng/ml, (c), or with FBS (10%, d). The representative level of thymidine incorporation (at the peak of DNA synthesis) was compared to non-treated cells (taken as 100%). Values are expressed as the means (%) +/- s.d. of quadruplicate wells from three separate experiments. (e) Total cell lysates from T47D cells were processed for Western blotting. Membranes were probed with anti-β<sub>1</sub>, anti-β<sub>4</sub>, or anti-α<sub>6</sub> antibodies.

### **FIG. 2. ECM affects the HRG-dependent phosphorylation of BRCA1.**

(a) Cells were seeded on plastic and starved for 48 h in growth medium containing 0.4% FBS. Four hours prior to stimulation with HRG (20 nM), cells were loaded with [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub>. After stimulation for the indicated times, total cell lysates were immunoprecipitated with anti-BRCA1 antibody, BRIN 129.5. Immunoprecipitates (IPs) were subjected to SDS-PAGE and incorporated radioactivity was visualized by autoradiography (upper panel). IPs were also subjected to Western blotting and membranes were probed with BRIH monoclonal anti-BRCA1 antibody (lower panel).

(b) Cells seeded on plastic (PL), laminin (LAM), poly-L-lysine (POL) or maintained in suspension (SUS) were starved, metabolically labeled with [<sup>32</sup>P], treated with HRG and lysed as indicated in Fig. 2A. Immunoprecipitates were processed the same way as indicated above for the upper and lower panels. (c) Cells grown on LAM coated plates or on PL were processed the same way as indicated above. Prior to stimulation with HRG, some plates coated with LAM were pre-treated with blocking anti-α<sub>6</sub> or blocking anti-β<sub>1</sub> antibodies alone or together, and processed as indicated above. Autoradiographs are representative of two independent experiments.

### **FIG. 3. Laminin enhances the HRG-dependent activation of the ErbB2 receptor and PI3 kinase.**

(a) Starved T47D cells seeded on plastic (PL), laminin (LAM), collagen type IV (COL), fibronectin (FN), poly-L-lysine (POL) or those maintained in suspension (SUS) were stimulated with HRG for 10 min. Total cell lysates were immunoprecipitated with anti-ErbB2 monoclonal antibody and then immunoprecipitates were subjected to Western blotting. PVDF membranes were probed with 4G10 antibody (upper panel), or after stripping, with an anti-ErbB2 antibody (lower panel). “C” indicates a sample of total cell lysates, from

stimulated cells grown on LAM, that was immunoprecipitated with protein-G-sepharose only. Some cells were treated with genestein (50  $\mu\text{g/ml}$ ) for 15 min prior to the 10-min treatment with HRG at the peak of tyrosine phosphorylation. As a comparison to the treatment with HRG, some cells were stimulated with FBS. (b) Starved cells, seeded on plastic (PL), laminin (LAM), collagen type IV (COL), or on fibronectin (FN) coated plates, were treated with heregulin (HRG, 20 nM) for 10 min. Total cell lysates were immunoprecipitated with either anti-phosphotyrosine antibody or with normal mouse IgG (IgG) for the *in vitro* kinase assay (b), or processed for Western blotting (c). (b) Immunoprecipitates were tested for their ability to phosphorylate lipids in the presence of  $[\gamma^{32}\text{P}]\text{ATP}$ . The phosphorylated lipids were then extracted from the reaction mixture by  $\text{CHCl}_3:\text{CH}_3\text{OH}$  [1:1] and spotted on TCL plates. The extracted, phosphorylated lipids were then separated in developing solution followed by autoradiography. (c) Immunoblots were probed with anti-p-Ser473 AKT antibody (\*p-AKT, upper panel) or with anti-AKT antibody (lower panel) after stripping of the membrane. Some cells were pretreated with LY294002 [(10  $\mu\text{M}$ , (b) and (c)] or with PD98059 [(50  $\mu\text{M}$ , (b) and (c)]. The autoradiograms are representative of two separate experiments. "C" = reaction mixture without cell lysates. PIP = phosphatidylinositol phosphates.

**FIG. 4. Laminin does not enhance the HRG-dependent activation of MAPK.**

T47D cells were seeded on plastic (PL), laminin (LAM), fibronectin (FN), collagen type IV (COL), or maintained in suspension (SUS). Starved cells were either left untreated or stimulated with heregulin (HRG, 20 nM) for 10 min. Obtained cytoplasmic cell lysates were either subjected to Western blotting (a) or immunoprecipitated with anti-Erk 1/2 antibody for the *in vitro* kinase assay (b). Some cells were treated with either PD98059 (50  $\mu\text{M}$ ) or with genestein (10  $\mu\text{M}$ ) prior to stimulation with HRG. (a) Blots of total cell lysates were probed with anti-phospho-Erk-2 antibodies (\*p-Erk, upper panel), and then stripped and reprobed with anti-Erk-2 antibody as a control for total Erk protein (lower panel). C = sample buffer + RIPA buffer. (b) Immunoprecipitates were used for the *in vitro* kinase assay with  $[\gamma^{32}\text{P}]\text{ATP}$  and myelin basic protein (MBP) as a substrate. Control (C) indicates the kinase activity of the sample containing lysate derived from cells treated with HRG that was mixed with protein-G agarose with no anti-Erk-2 antibody. The lower panel represents immunoblots of immunoprecipitates probed with anti-Erk-2 antibody. Autoradiograms are representative of two independent experiments.

**FIG. 5. Effect of HRG and/or ECM on BRCA1 mRNA expression in T47D and HCC-1937 cells.**

Cells were seeded on plastic (a) or on plates coated with laminin (b), or collagen type IV (c) and then starved for 48 h. Cells were next treated with HRG (20 nM, H) or with DMSO (D). At the indicated times, total RNA was collected using a Qiagen kit and 20  $\mu\text{g}$  of the total RNA was subjected to Northern blotting. (d) HCC-1937 and T47D cells were seeded on plastic (PL), laminin (L) or on collagen type IV (C) and starved for 48 h as indicated in Fig. 2. Total RNA was collected and processed the same way as described above. Blots in (a), (b),

(c), and (d) were probed with an 1179-bp fragment of BRCA1 (upper panels) and with actin cDNA or 18S rRNA as indicated (lower panels). Results are representative of two independent experiments.

**FIG. 6. Effect of various inhibitors on the level of BRCA1 mRNA in T47D breast cancer cells.**

T47D cells were seeded on plastic plates [(a), (b), (c) and (d)] and starved in growth medium containing 0.4% FBS. After 48 h, cells were either untreated (0) or treated with HRG alone (a) or with HRG in the presence of actinomycin-D (b) or cycloheximide (c) for the indicated times. (d) Some cells were also treated with ALLN or lactacystin, alone or in combination, prior to the treatment with HRG for 4 h. Total RNA was extracted and processed the same way as indicated in Fig. 5. Autoradiograms are representative of two independent experiments.

**FIG. 7. Effect of HRG, ECM and various inhibitors on BRCA1 protein level.**

T47D cells seeded on plastic [(a) and (b)], collagen type IV (c) or on laminin (d) coated plates were starved and treated with HRG (20 nM) [(b), (c) and (d)] or with DMSO [(a) and (b)] as a control for the indicated times (hours, h). (a) Total cell lysates were collected and subjected to Western blotting. Membranes were probed with anti-BRCA1 antibody (upper panel) or, after stripping, with anti-actin antibody (lower panel). (b) After cells were treated with HRG or with DMSO, total cell lysates were collected at the indicated times and subjected to Western blotting. Membranes were probed with anti-BRCA1 antibody (upper panel) or, after stripping, with anti-ErbB2 antibody (lower panel). [(c) and (d)] T47D cells were seeded on collagen type IV (20 µg/ml, C) or on laminin (20 µg/ml, d) coated plates, starved, and then stimulated with HRG for the indicated times. Total cell lysates were processed the same way as indicated in Fig. 7B. Membranes were stripped and re-probed with anti-actin antibodies. The blots are representative of three independent experiments.

**FIG. 8. Effect of various inhibitors on BRCA1 protein level.**

(a) Cells seeded on laminin (L), collagen type IV (C) or on plastic (PL) were treated with HRG for the indicated times (H). Some cells grown on PL were pre-treated with PSI, before the addition of HRG. Total cell lysates were processed as described in Fig. 7A. (b) Starved T47D cells seeded on plastic were treated with HRG in the presence of cycloheximide (CHX) for the indicated times and total cell lysates were analyzed. (c) Cells were treated with HRG and actinomycin-D (Act-D). The lower panels indicate the level of ErbB2 (a) or actin (b) and (c), after re-probing the membranes with the corresponding antibodies. The blots of all panels are representative of two independent experiments.

**FIG. 9. Forced expression of PTEN suppresses HRG-dependent Akt phosphorylation**

MDA-MB-231 cells were seeded into 60-mm petri dishes and starved for 48 h. Some plates were subjected to forced expression of wild type PTEN (WT), PTEN inactive mutant (C/S), PTEN with non-meaningful mutation



[(G/R), (all containing HA tag)], or as a control with empty vector (V). After transient expression of PTEN constructs (24 h) cells were left non-treated (0), or treated with heregulin (H, 20 nm) for 5 min and total cell lysates were subjected to immunoprecipitation with monoclonal anti-PTEN antibody. Portions of total cell lysates (TCL) and immunoprecipitates were run on SDS-PAGE, transferred onto PVDF membrane and probed with polyclonal anti-phosphoAkt, monoclonal anti-Akt or monoclonal anti-HA antibodies. The signal from separated proteins was detected by autoradiography after subjecting the membranes to ECL.

## **APPENDIX A**

### **Figures**

Fig. 1

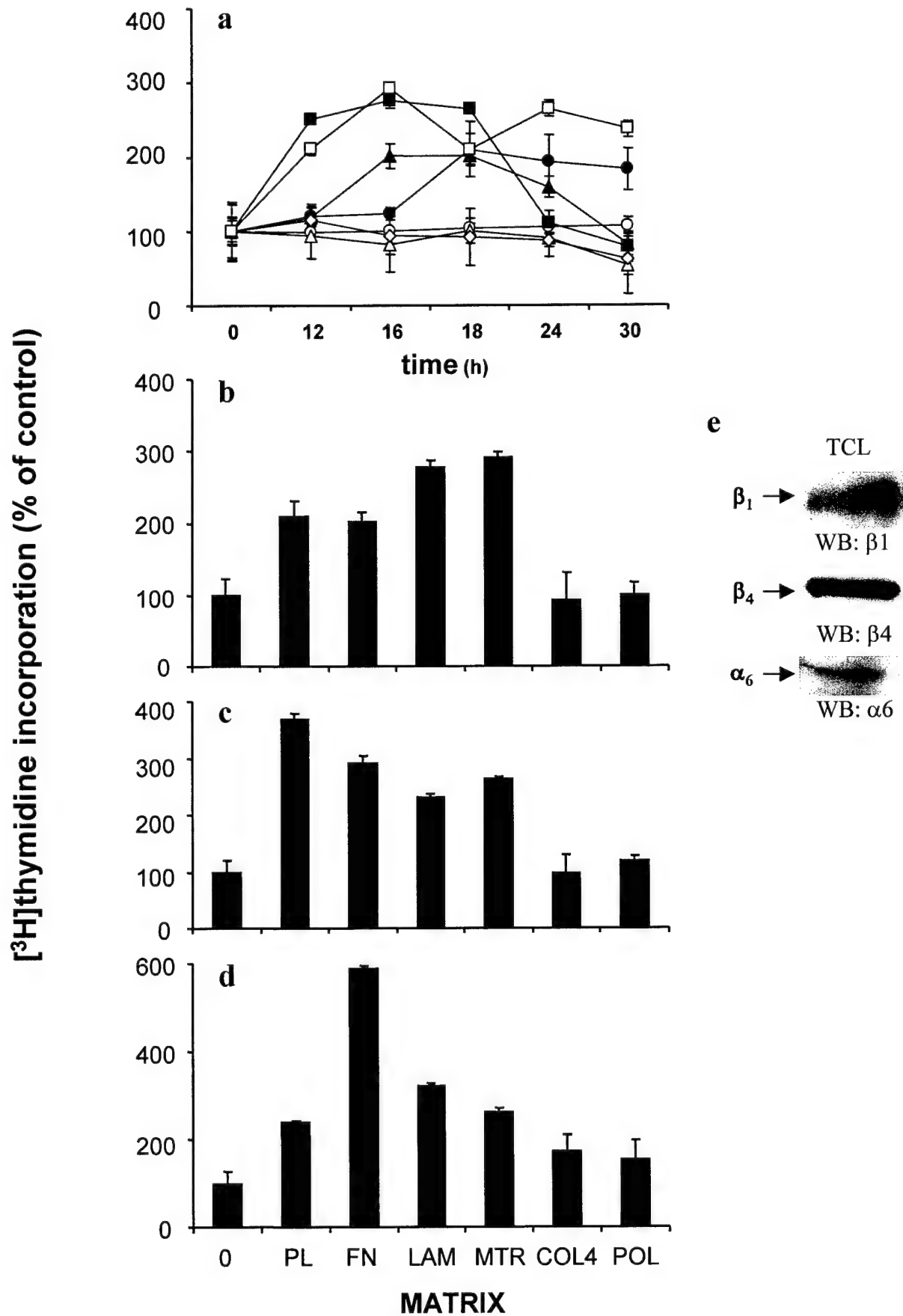


Fig. 2

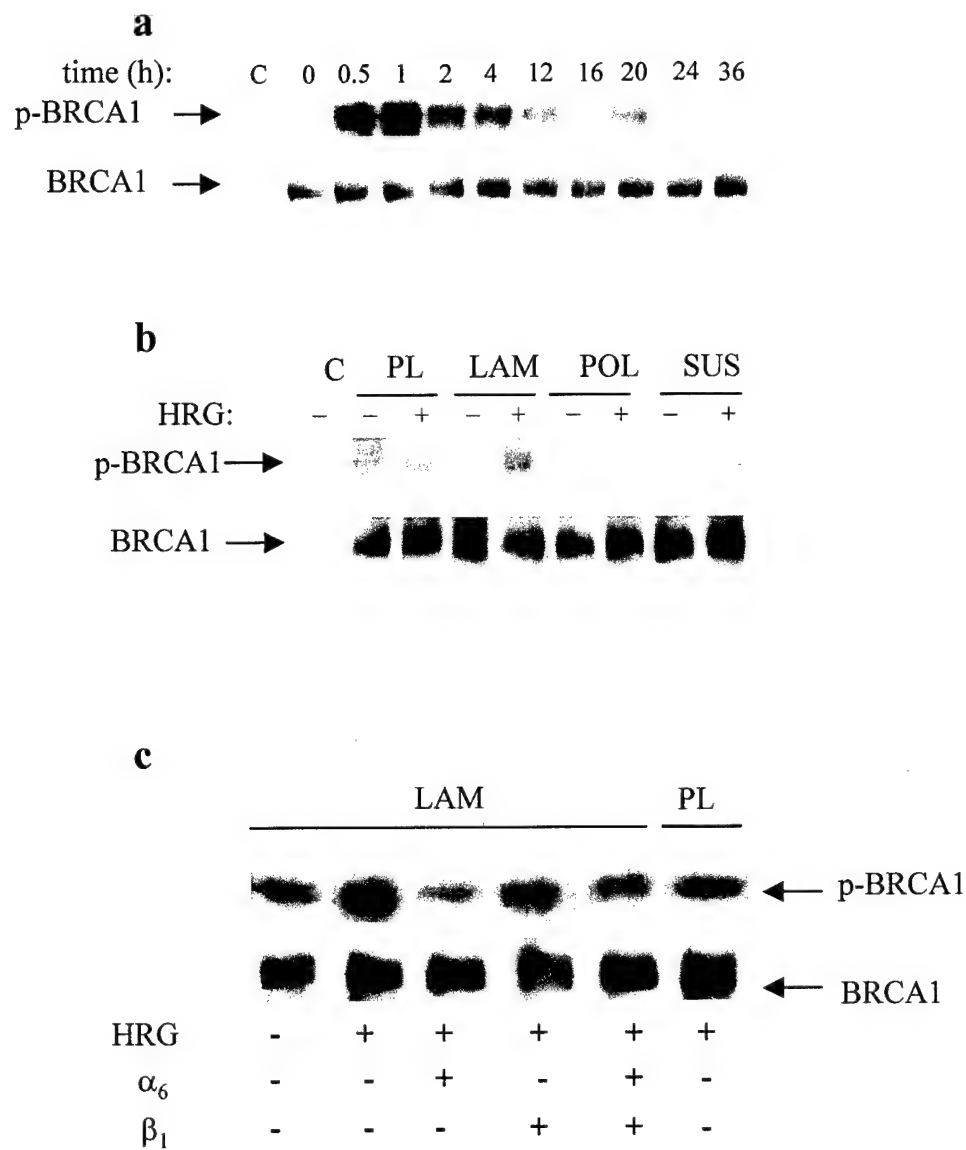


Fig. 3

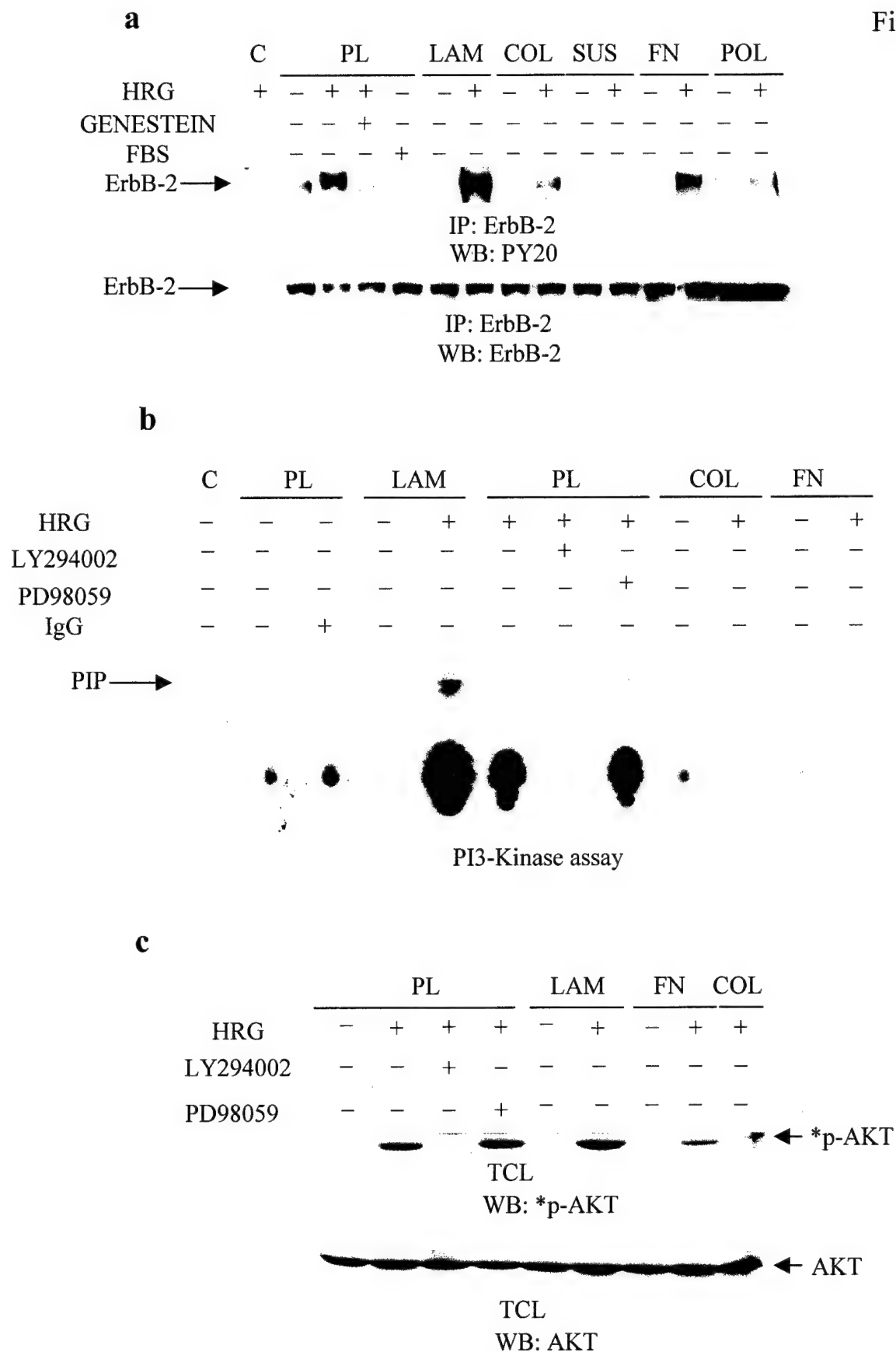


Fig. 4

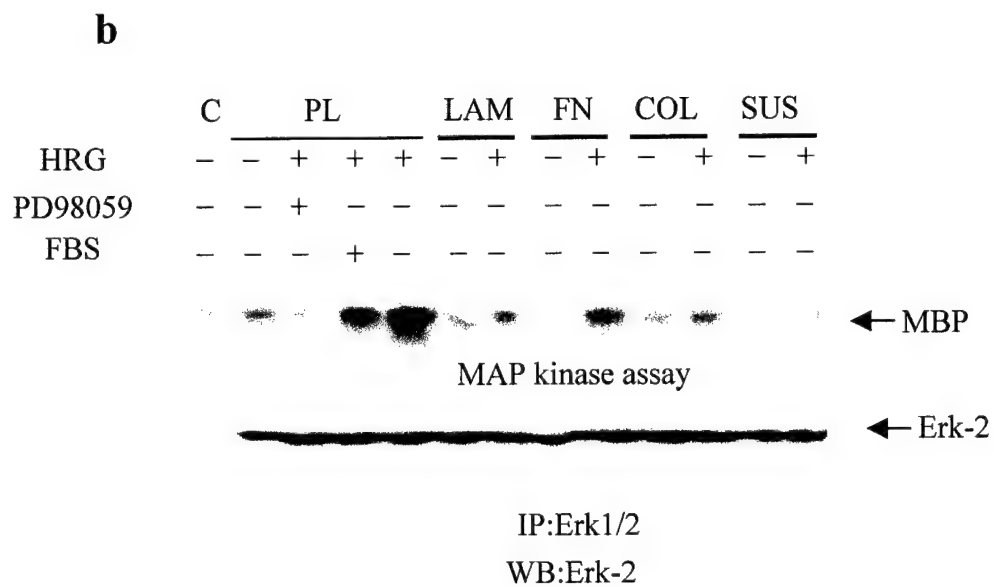
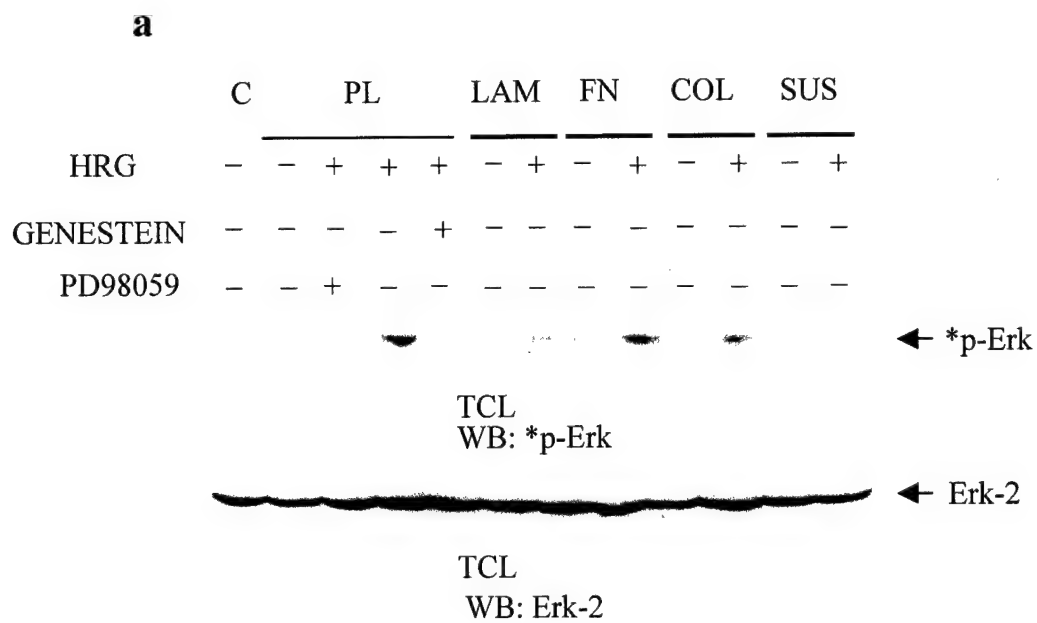


Fig. 5

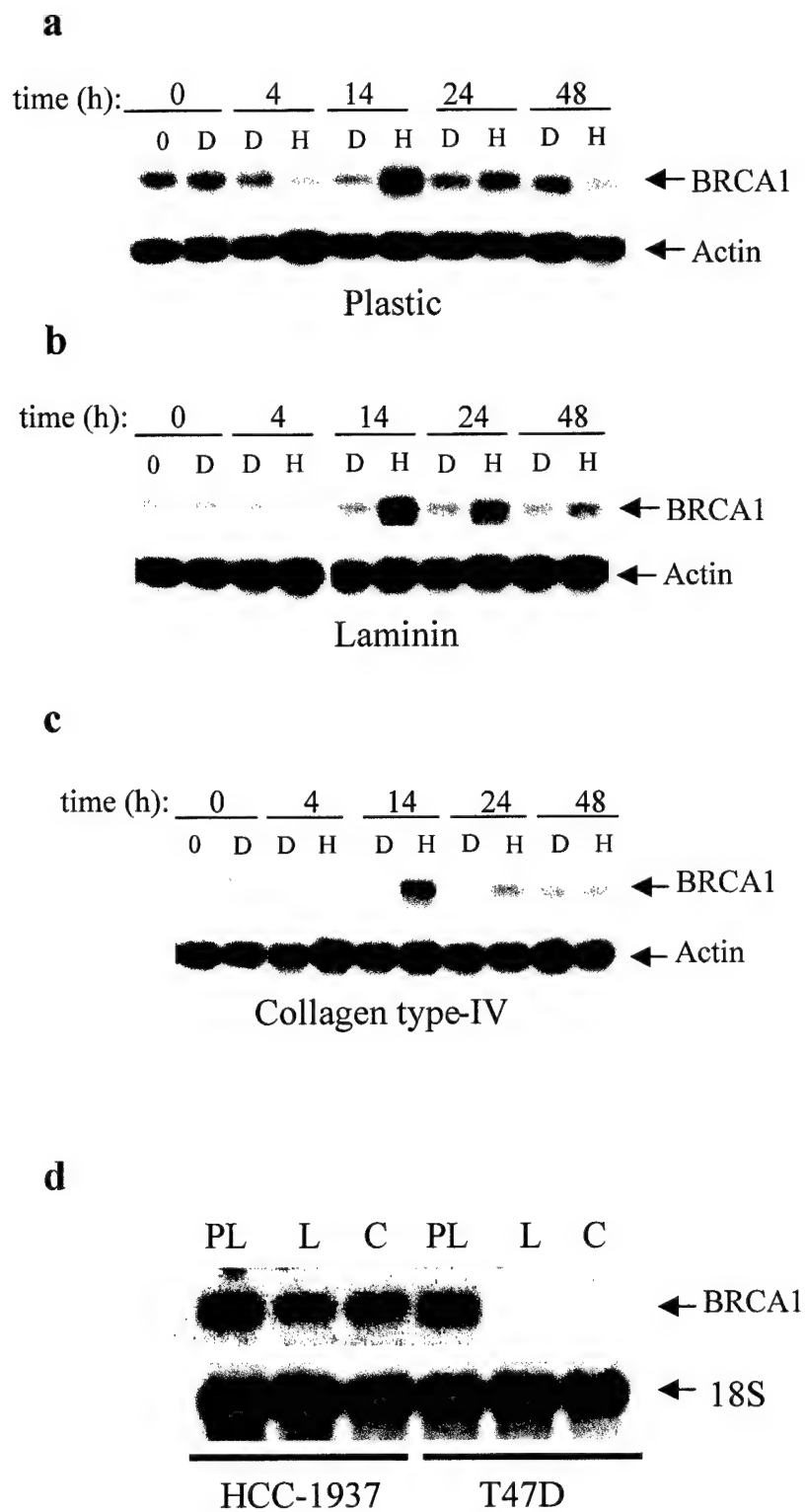
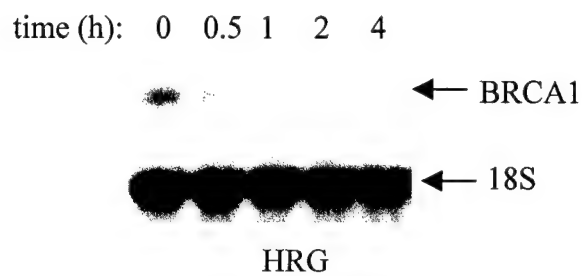


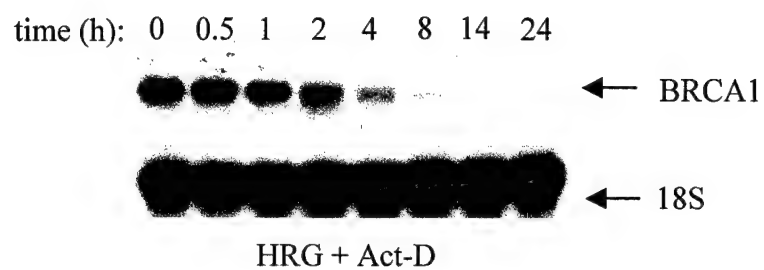


Fig. 6

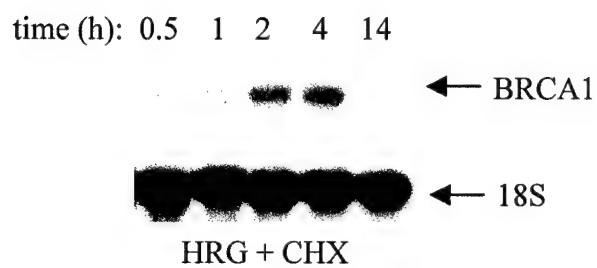
**a**



**b**



**c**



**d**

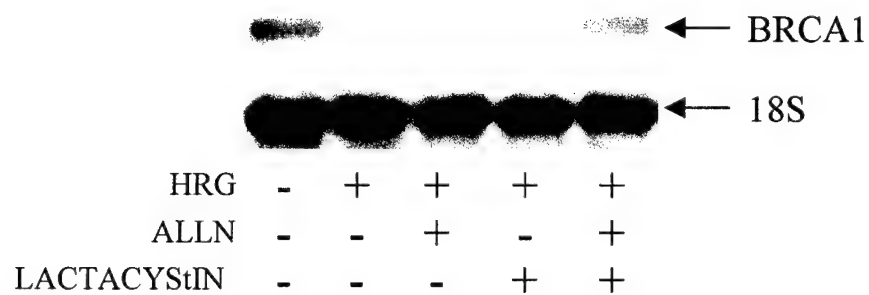
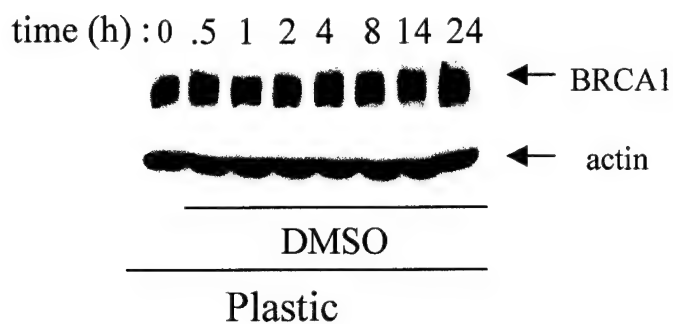
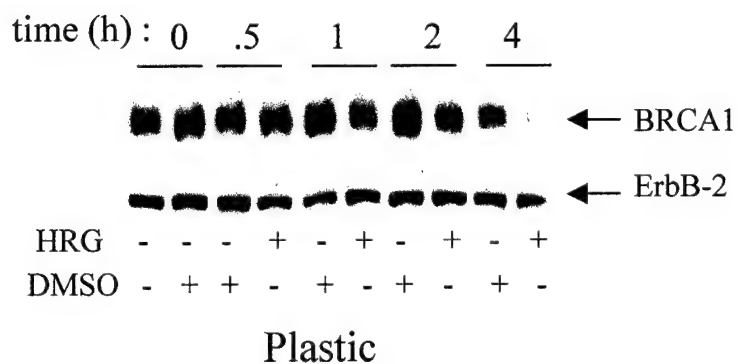


Fig. 7

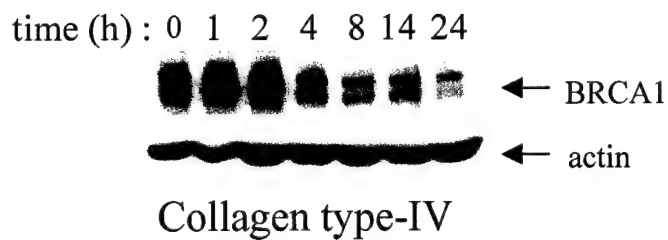
**a**



**b**



**c**



**d**

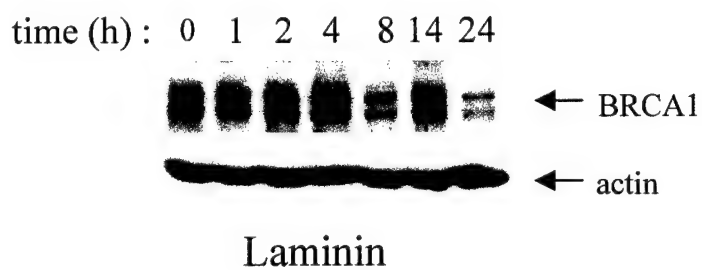


Fig. 8

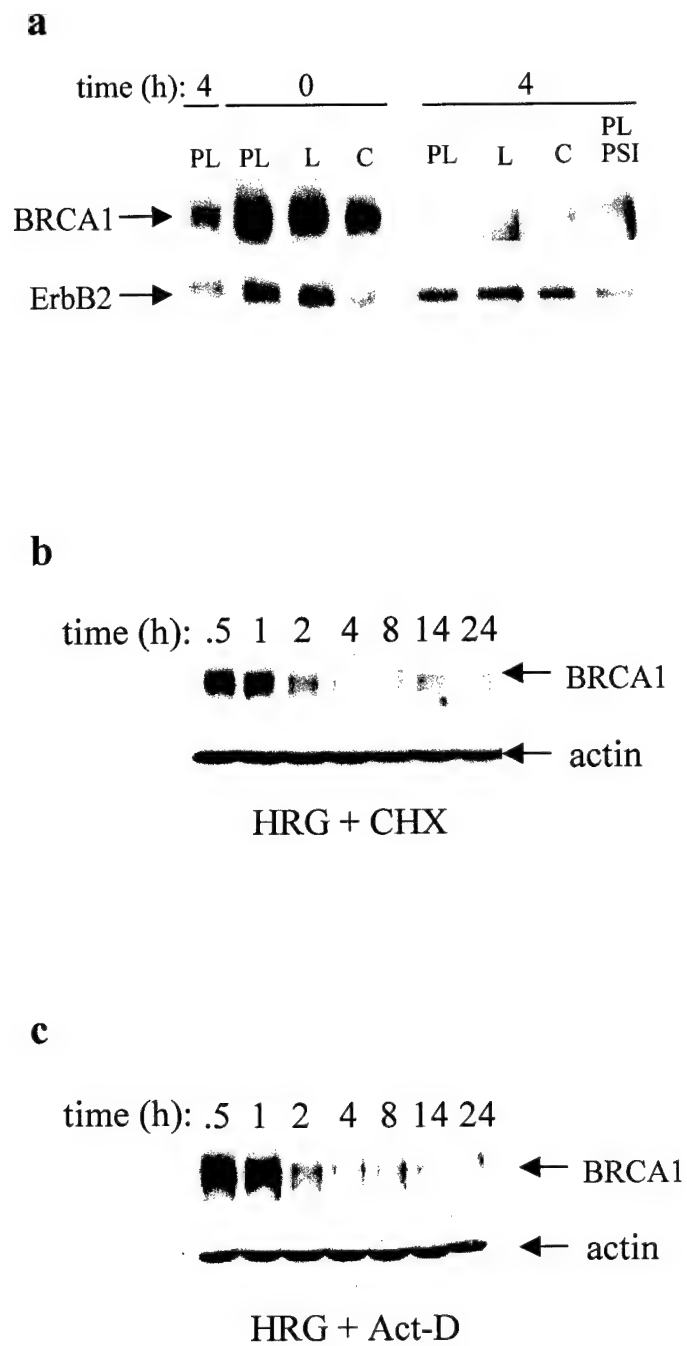
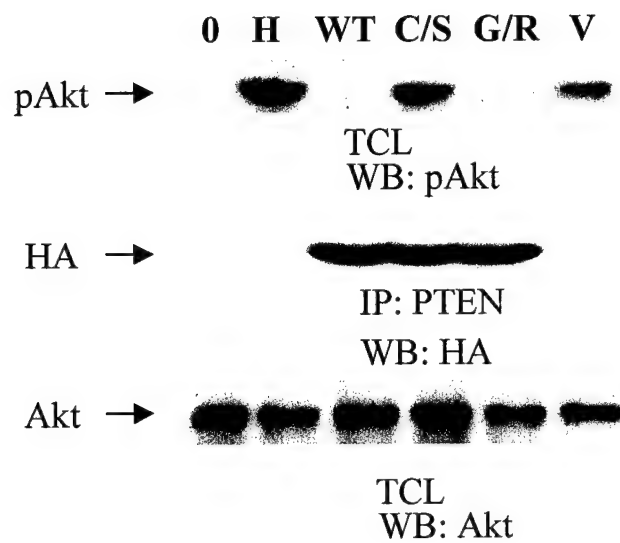


Fig. 9



## **APPENDIX B**

**Poster presented at AACR meeting**

However, in MDA-MB-435 cells, the levels and activity of Rac are higher in the maspin transfectants. This suggests that the two cell lines may utilize different mechanisms to regulate cell motility. Maspin expression in MDA-MB-231 cells may interfere with cell motility by down-regulation of Rac1 and Rac activity. Thus, the unique phosphorylation of maspin may be integral to the initiation of its involvement in the regulation of breast cancer motility properties, which may provide new therapeutic approaches for using maspin to inhibit tumor invasion and metastasis.

**#5178 Calcium regulation of matrix metalloproteinase 2-mediated migration in oral squamous carcinoma cells.** Hidayatullah G. Munshi, Yi Wu, Edgardo Ariztia, and Mary Sharon Stack. *Northwestern University, Chicago, IL.*

Activation of matrix metalloproteinase 2 (MMP-2) has been shown to play a significant role in the behavior of cancer cells, affecting both migration and invasion. The activation process requires multimolecular complex formation between proMMP-2, membrane type 1-MMP (MT1-MMP) and tissue inhibitor of metalloproteinases-2 (TIMP-2). Because calcium is an important regulator of differentiation in normal keratinocytes, we evaluated the effect of calcium on MMP regulation in two oral squamous cell carcinoma lines (SCC25 and SCC15), as well as in SCC25 overexpressing MT1-MMP (SCC25-MT). Increasing extracellular calcium (0.09-1.2 mM) resulted in a dose-dependent increase in MT1-MMP-dependent proMMP-2 activation. Despite the requirement of MT1-MMP in the activation process, no changes in MT1-MMP expression, post-translational processing or cell surface localization were apparent, although an inverse relationship between proMMP-2 activation and TIMP-2 levels in conditioned media was observed. Alterations in TIMP-2 levels in the conditioned media were prevented by a broad-spectrum MMP inhibitor, suggesting that the decline in TIMP-2 with increase in extracellular calcium may be due to enhanced MMP-dependent degradation of TIMP-2. In functional studies, increasing calcium enhanced cellular migration on laminin-5-rich matrix using an *in vitro* colony dispersion assay. Migration was MMP-dependent, as the addition of a broad-spectrum MMP inhibitor or function blocking MT1-MMP antibody decreased motility. Taken together, these results suggest that changes in extracellular calcium can regulate MMP-2 activation and thus affect the cellular behavior of oral squamous cell carcinoma cells.

**#5179 Metastasis dormancy in colon carcinoma.** Neelam Todi Shah, Julio Aguirre Ghiso, Yeri Estrada, and Liliana Ossowski. *Mount Sinai Medical Center, NY, NY.*

Cancer metastasis can be clinically undetectable for years after curative resection suggesting that disseminated cells may remain dormant for extended periods of time. This scenario is also seen in patients with colon cancer. The premise that prolonging metastasis dormancy will prolong life provided the impetus for exploring mechanisms of dormancy. We showed evidence that downregulation of urokinase plasminogen activator receptor (uPAR) renders human epidermoid carcinoma cells dormant. In contrast, when uPAR is highly expressed, it interacts with and activates  $\alpha 5 \beta 1$  integrin leading to activation of the mitogenic ERK pathway. The active integrins assemble fibronectin (FN) fibrils which inhibit the growth suppressive p38 pathway. Thus, a balance between ERK and p38 activities determines tumorigenicity or dormancy and the balance can be shifted towards ERK by inhibitors of p38. We tested whether similar mechanisms regulate tumorigenicity of colon cancer cells and found that of five cell lines evaluated including Colo205, SW620, HT29, HCT15 and HCT116, only HCT116 had detectable (but not highly expressed) uPAR, and expressed  $\alpha 5 \beta 1$  integrin. We examined ERK activity and found that SW620, which had mutated RAS, expressed the highest levels of ERK activation which correlated with their rapid *in vivo* growth. We also tested whether a negative cross talk between p38 and ERK activities, found in the epidermoid carcinoma, existed in colon cancer cells. Three of the five cell lines incubated in culture with p38 inhibitor demonstrated activation of ERK. We will test whether this activation corresponds to increased tumorigenicity of colon carcinoma *in vivo*. We conclude that in colon carcinomas ERK and p38 activities are independent of uPAR expression and  $\alpha 5 \beta 1$  activation and that other upstream signals, including mutated RAS, may be responsible for the ERK/p38 balance and regulation of tumorigenicity and dormancy.

**#5180  $\alpha v$  integrins-mediated proliferation of human ovarian adenocarcinoma cells by regulation of p27Kip1 through the ILK/Akt pathway.** Severine Cruet-Hennequart, Sylvie Maubant, Pascal Gauduchon, and Shoukat Dedhar. *Jack Bell Research Centre, Vancouver, BC, Canada, and Groupe Regional d'Etudes sur le Cancer, Caen, France.*

The aim of this study was to determine if  $\alpha v$  integrins were involved in ovarian carcinoma cells proliferation. An anti- $\alpha v$  blocking antibody specifically inhibited the growth of two human ovarian carcinoma cell lines, IGROV1 and SKOV-3, despite the presence of fetal bovine serum. This effect was linked to reduced adhesion and spreading in IGROV1 but only to altered spreading in SKOV-3. Using specific anti- $\alpha v \beta 3$  or anti- $\alpha v \beta 5$  antibodies, we showed that the effects were specific of  $\alpha v \beta 3$  in IGROV1 cells, and involved both  $\alpha v \beta 3$  and  $\alpha v \beta 5$  in SKOV-3 cells. The reduced growth rate induced by  $\alpha v$ -integrin blockade was linked in both cell lines to a decrease in cell number in S and G2/M phases. Interestingly, the decrease in cell growth through  $\alpha v$  integrin blockade by antibodies, was accompanied by an inhibition of phosphorylation of PKB/Akt on serine-473 and in an increase in p27Kip1 expression. The phosphorylation of PKB/Akt on serine-473

appeared to be regulated especially by  $\alpha v$  integrins in PTEN-negative (or low level) ovarian cells since it was inhibited through  $\alpha v$  integrin blockade whereas it was constitutive after serum deprivation. Moreover, inhibition of Integrin-Linked Kinase (ILK) activity using a pharmacological inhibitor also resulted in the same effects, which suggests that  $\alpha v$  integrins regulate the S phase progression of human ovarian carcinoma cells by regulating p27Kip1 through the ILK/Akt pathway.

**#5181 The small G protein, ras, regulates the glycosylation of the  $\beta 1$ , but not the  $\beta 3$ , integrin cell adhesion receptor.** Eric Seales, Anuj Singhal, and Susan Bellis. *University of Alabama at Birmingham, Birmingham, AL.*

The abnormal function of integrin cell adhesion receptors contributes to multiple processes related to tumorigenesis and/or metastasis, including increased cell invasiveness and anchorage-independent growth. Accordingly, intense investigation has centered on understanding the regulation of these receptors. In the present study we propose that variant N-glycosylation represents an important mechanism for the regulation of  $\beta 1$  integrins, but not  $\beta 3$  integrins. Our results show that the expression of an oncogenic (constitutively active) ras isoform in HD3 colon epithelial cells causes increased a2-6 sialylation of  $\beta 1$  integrins, whereas expression of a dominant negative ras isoform induces decreased a2-6 sialylation, relative to cells with wild-type ras. In contrast,  $\beta 3$  integrins do not carry a2-6-linked sialic acid residues, regardless of the state of ras activation. Cells with differentially a2-6 sialylated  $\beta 1$  integrins demonstrate altered cell adhesion to, and spreading on, collagen I, a  $\beta 1$ -specific integrin ligand. Conversely, cell binding and spreading on vitronectin, a  $\beta 3$ -specific ligand, are not altered by changes in ras activity. Further supporting our hypothesis that sialylation of the  $\beta 1$  integrin plays a critical role in the ligand/receptor interaction, cell binding to collagen I is inhibited by treating cells with the sialic acid-cleaving enzyme, sialidase. In contrast, sialidase treatment had no effect on cell binding to vitronectin. Collectively, our results suggest that the level of ras activity directly regulates the degree of a2-6 sialylation on the  $\beta 1$  integrin. In turn, differentially-sialylated  $\beta 1$  integrins have altered affinity for  $\beta 1$  integrin ligands such as collagen I. Ras does not affect the glycosylation or function of  $\beta 3$  integrins. Thus, cells with oncogenic ras (and hence, differentially-sialylated  $\beta 1$  integrins) have decreased affinity for collagen, relative to wild type cells, but sustained affinity for vitronectin. This ras-dependent change in cell preference for matrix molecules could contribute to the invasive behavior of tumor cells.

**#5182 Extracellular matrix affects the heregulin-dependent phosphorylation and expression of BRCA1 in breast cancer cells.** Tihomir Miralem and Hava Avraham. *Beth Israel Deaconess Medical Center/HIM, Boston, MA.*

BRCA1 is a nuclear protein that is phosphorylated in a cell cycle- and heregulin (HRG)-dependent manner. The HRG-dependent phosphorylation of BRCA1 is mediated through the PI3K/AKT pathway and involves phosphorylation of the Thr<sup>299</sup> residue located in the nuclear translocation signal. Since altered interaction between cells and surrounding extracellular matrix (ECM) is a common feature in a variety of tumors, we hypothesized that ECM may affect the HRG-dependent expression and phosphorylation of BRCA1. Following stimulation by HRG (20 nM), a strong increase in [<sup>3</sup>H]thymidine incorporation, peaking between 12 and 18 h, was observed in cells seeded on plastic (PL), suggestive of cells passing through S-phase. When T47D cells were seeded on laminin (LM) or matrigel, HRG caused a significantly higher mitogenic response than in those cells seeded on plastic. Furthermore, T47D cells seeded on poly-L-lysine (POL) or on collagen IV (CL4) did not respond at all, suggesting integrin involvement in this process. In support of this, we found that T47D cells do express beta1, beta4 and alpha6 integrins. HRG treatment also caused a transient phosphorylation of BRCA1, peaking between 0.5-1 h, in T47D cells plated on PL. Interestingly, when these cells were grown on LM, the level of BRCA1 phosphorylation was significantly increased, while the phosphorylation of BRCA1 was much lower in cells seeded on POL or in cells maintained in suspension. Accordingly, cells grown on LM had the greatest increase in ErbB2 activation, PI3K activity, and phosphorylation of AKT. When the effect of ECM on the expression level of BRCA1 was tested, we observed a similar pattern of mRNA expression on PL, LM and CL4. However, there was a significant decrease in the steady state of the BRCA1 mRNA level on both matrices (LM, CL4) as compared to cells seeded on PL. Likewise, there was a strong decrease in the protein level of BRCA1 at 4 h after treatment with HRG as compared to the BRCA1 level of non-treated cells. Thus, we concluded that ECM affects HRG-dependent BRCA1 expression at the mRNA and protein levels as well as the HRG-dependent phosphorylation of the BRCA1 protein.

**#5183 Hyposialylated  $\beta 1$  integrins with enhanced fibronectin-binding capability are expressed during myeloid cell differentiation.** Alexis C. Semel, Eric C. Seales, Anuj Singhal, and Susan L. Bellis. *University of Alabama at Birmingham, Birmingham, AL.*

During phorbol ester (PMA)-induced differentiation of myeloid cells along the monocyte/macrophage lineage, cells become highly adherent to fibronectin. To elucidate the mechanisms that underlie increased cell adhesiveness, we assayed integrin cell adhesion receptors for potential changes in structure and function following PMA treatment. Our results show that PMA treatment of U937 and THP-1 myeloid cells induced a marked increase in cell attachment to fibronectin that was dependent upon  $\beta 1$ , but not  $\beta 2$  or  $\beta 3$  integrin receptors. Coincident with increased fibronectin binding,  $\beta 1$  integrins demonstrated altered electrophoretic

## **APPENDIX C**

**Manuscript submitted to MCB**



# **Extracellular Matrix Enhances Heregulin-Dependent BRCA1 Phosphorylation and Suppresses BRCA1 Expression Through its C-Terminus**

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Running title: Effect of ECM on BRCA1

Key Words: BRCA1, Extracellular matrix, heregulin, ErbB-2, breast cancer, integrins

## Abstract

Germline mutations in the breast cancer susceptibility gene BRCA1 account for the increased risk of early onset of familial breast cancer, whereas overexpression of the ErbB family of receptor tyrosine kinases has been linked to the development of non-familial or sporadic breast cancer. To analyze whether there is a link between these two regulatory molecules, we studied the effects of ErbB-2 activation by HRG on BRCA1 function.

We have previously demonstrated that HRG induced the phosphorylation of BRCA1, which was mediated by the phosphatidylinositol-3 kinase (PI3K)/Akt pathway. Since altered interaction between cells and surrounding extracellular matrix (ECM) is a common feature in a variety of tumors and since ECM modulates intracellular signaling, we hypothesized that ECM may affect the expression and HRG-dependent phosphorylation of BRCA1. Following stimulation by HRG, a strong increase in [<sup>3</sup>H]thymidine incorporation was observed in human T47D breast cancer cells seeded on plastic (PL). When T47D cells were seeded on laminin (LAM) or matrigel, HRG induced a significantly higher mitogenic response as compared to cells seeded on plastic. Furthermore, T47D cells seeded on poly-L-lysine (POL) had an abrogated mitogenic response, indicating the involvement of integrins in this process. HRG treatment induced a transient phosphorylation of BRCA1 that was enhanced in T47D cells grown on LAM. LAM-enhanced BRCA1 phosphorylation was mediated through  $\alpha_6$  integrin. Accordingly, T47D cells grown on LAM had the greatest increase in ErbB-2 activation, PI3K activity and phosphorylation of Akt. A similar pattern of BRCA1 mRNA expression was observed when T47D cells were seeded on PL, LAM or CL4. There was

a significant decrease in the steady state of the BRCA1 mRNA level on both LAM and CL4 matrices as compared to cells seeded on PL. Interestingly, BRCA1 mRNA expression in HCC-1937 breast cancer cells, which express C-terminus truncated BRCA1, was not affected by either LAM or CL4. In addition, HRG stimulation caused a significant decrease in BRCA1 mRNA expression that was dependent on protein synthesis. Pretreatment with both the calpain inhibitor, ALLN, and the proteasome inhibitor, lactacystin, inhibited the HRG-induced down-regulation of BRCA1 mRNA expression. Likewise, there was a strong decrease in the protein level of BRCA1 in T47D cells 4 h after treatment with HRG as compared to its level in control non-treated T47D cells. Pretreatment with the proteasome inhibitor, PSI, inhibited also the HRG-induced down-regulation of BRCA1 protein in breast cancer cells. These findings suggest that ECM enhances HRG-dependent BRCA1 phosphorylation and that ECM and HRG down-regulate BRCA1 expression in breast cancer cells. Furthermore, ECM suppresses BRCA1 expression, which was mediated through the C-terminus of BRCA1.

## Introduction

BRCA1 is a tumor suppressor gene whose inactivation is associated with a high incidence of familial breast and ovarian cancers (5). Human BRCA1 encodes an 1863-amino-acid nuclear phosphoprotein (55, 58). BRCA1 mRNA and protein levels are cell cycle regulated (71, 76) through the balance between proteolytic degradation and transcription (6). BRCA1 contains a RING finger domain at its amino terminus and two BRCT domains at its C-terminus (34). BRCA1 alone, (41) and together with BARD1 (27), promotes ubiquitin polymerization while loss of BRCA1 ubiquitin protein ligase activity was linked to its radiation protection function (54).

BRCA1 was implicated as a caretaker gene, having a role in controlling recombination and genome integrity through association with Rad51 (59), and in transcription-coupled repair of oxidative DNA damage (20). The C-terminal domain of BRCA1 has transcriptional activation activity, and is linked to the RNA polymerase II holoenzyme via RNA helicase A (3, 47). BRCA1 was reported to be associated with proliferation (64), differentiation (42) and apoptosis (60).

BRCA1 protein is phosphorylated in response to DNA damage (39), in a cell cycle-dependent manner (55), after treatment with heregulin (2) and after association with casein kinase 2 (48). While DNA damage-dependent phosphorylation of BRCA1 is elicited by ataxia telangiectasia mutated kinase (ATM) (19) and by hCds1 (37), its cell-cycle-dependent phosphorylation is elicited through CDK2 (53). BRCA1 contains four CDK consensus sites (53) and interacts with cyclin-D (73) and with CDK4 (73). The HRG-dependent phosphorylation of BRCA1 was mediated at its nuclear translocation domain through a PI3K/Akt-dependent pathway (2).

The basement membrane (BM), as a component of extracellular matrix (ECM), is composed of laminin, collagen type IV and heparan sulfate proteoglycans (66). It interacts with cells through integrins and thereby regulates cell cycle progression, movement, survival, gene expression, and physical support (29). Integrins are heterodimeric proteins formed from  $\alpha$  and  $\beta$  subunits, and each  $\alpha\beta$  combination confers a specific binding property to the cells (29). For instance,  $\alpha_6$  and  $\beta_{1,4}$  are specific for laminin, while  $\alpha_{1,2,3}$  and  $\beta_{1,2}$  are specific for collagen type IV (40). Integrins functionally can modulate receptor tyrosine kinases. EGFR auto-phosphorylation is enhanced in a number of cell types, including fibroblasts, smooth muscle, and kidney epithelial cells upon their interaction with ECM proteins (11, 31, 46). In ovarian and breast cancer cells,  $\alpha_6\beta_4$  integrin co-immunoprecipitated with ErbB2. Ligation of this integrin increased ErbB2 phosphorylation and invasion through the activated PI3K/Akt pathway (18). ECM including the basement membrane, through integrins (26), regulate numerous intracellular signaling molecules including GTPases, cytoplasmic kinases and growth receptor tyrosine kinases in breast cancer cells (29). In this regard, the cytoplasmic kinases, such as MAPK and PI3K/Akt, were activated by the binding of laminin to either  $\alpha_6\beta_1$  or  $\alpha_6\beta_4$  integrin, respectively (61, 75). Furthermore, integrins play a major role in increasing cell tumorigenicity by stimulating the invasion of breast and ovarian cancer cells through PI3K and activation of tyrosine kinases (18). In progressive breast cancer, an initial, non-tumorigenic cell phenotype is followed by the loss of basement membrane (BM)-cell interactions (9), which is associated with ErbB-2 overexpression (56) and decreased BRCA1 expression (69). This leads to a phenotypic switch where cancer cells become tumorigenic, as manifested by their hyperproliferation and increased

invasiveness (69). In the present study, we have elucidated the effects of extracellular matrix on BRCA1 expression and its role in modulating HRG-dependent BRCA1 phosphorylation and expression.

## MATERIAL AND METHODS

**Materials.** Antibodies used for immunological analysis were as follows: anti-BRCA1 antibody and anti-phosphotyrosine antibody (4G10) were from Genentech (San Francisco, CA). Phospho-Erk (E-4) antibody, anti-Erk-2 (C-154), anti- $\beta_4$  integrin, anti-ErbB-2 (Neu) and HRP-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt and anti-phospho-Ser-473 Akt antibodies were from New England-Biolabs (Beverly, MA). Anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). Monoclonal anti-actin antibody was from Chemicon (Temecula, CA). BRCA1 monoclonal antibodies N-129.5 and H-945.2 were kindly provided by Beckman Coulter, Inc. Genestein, LY294002, actinomycin-D, N-acetyl-Leu-Leu-Norleucinal (ALLN) and phosphatidylinositol were from Sigma Chemical Co. (St. Louis, MO). PD98059, cycloheximide and lactacystin were from Calbiochem-Novobiochem Co. (La Jolla, CA). EGF and myelin basic protein (MBP) were from Upstate Biotechnology (Lake Placid, NY) and heregulin (HRG) was from Genentech.  $\gamma$ [ $^{32}\text{P}$ ]ATP,  $\alpha$ [ $^{32}\text{P}$ ]dCTP and [ $^3\text{H}$ ]thymidine were from New England Nuclear (Boston, MA). Blocking anti- $\beta_1$  integrin antibody was from Hybridoma Bank (University of Iowa, Iowa City, IA) and blocking anti- $\alpha_6$  integrin antibody was a generous gift from Leslie Shaw (Beth Israel Deaconess Medical Center, Boston, MA). N-benzyloxycarbonyl-Ile-Glu-(*O*-t-butyl)-Ala-leucinal (PSI) was from Sigma. All other chemicals were from Fisher Scientific (Norcross, GA), unless otherwise specified.



**Cell culture.** T47D cells were obtained from ATCC. These cells were cultured in RPMI-1640 medium (Gibco-BRL), supplemented with 3.5  $\mu\text{g/ml}$  insulin, 10% fetal bovine serum (FBS, Gibco-BRL), and penicillin/streptomycin. HCC-1937 breast cancer cells contain a truncated BRCA1 C-terminal and therefore express non-functional BRCA1 protein (8). They were cultured in Iscove's medium (Gibco-BRL), supplemented with 15% FBS and penicillin/streptomycin. To coat the plates, mouse laminin (20  $\mu\text{g/ml}$ ), human plasma fibronectin (30  $\mu\text{g/ml}$ ), collagen type IV (40  $\mu\text{g/ml}$ ) and Matrigel (Engelberth-Holm-Swarm tumor basement membrane, 40  $\mu\text{g/ml}$ ) (Becton-Dickinson, Bedford, MA) were dissolved in sterile water, 0.05 M HCl, or in cold medium, respectively, spread on culture dishes (1.0 ml/10-cm petri dish, 70  $\mu\text{l/well}$  in 24-well plates), and allowed to dry in a sterile environment. Collagen type I solution (2.9 mg/ml) was purchased as Vitrogen-100 (Cohesion, Palo Alto, CA) and was added to the wells of the tissue culture plates (0.25 ml/well in 24-well plates), then incubated for at least 60 min at 37°C to gel. Poly-L-lysine (40  $\mu\text{g/ml}$ , Sigma) was dissolved in water spread on culture dishes (70  $\mu\text{l/ml}$  in 24-well plates) and allowed to dry in a sterile environment. Quiescence was induced by replacing the growth medium of cells at 60-80% confluence with medium containing 0.4% FBS, then followed by 48 h incubation. To study the initiation of signaling, cells were stimulated with either HRG (20 nM), FBS (10%), EGF (10 ng/ml) or with DMSO (1:1000 dilution). To maintain cells in suspension, starved cells were detached by trypsinization, resuspended in 15 ml of growth medium, placed into an Erlen-Mayer flask containing a stirring bar, and then placed into a humidified incubator for 30–45 min. For metabolic loading, sub-confluent cultures of T47D cells grown on 100-mm coated or non-coated Corning tissue culture dishes were preincubated

for 3 h at 37°C in RPMI-1640 phosphate-less medium containing 100  $\mu$ Ci/ml of carrier-free [ $^{32}$ P]orthophosphate (HCl free) before the addition of agonist.

**Cell attachment.** Cell attachment was performed as described (45). Briefly, labeled or unlabeled cells were released from petri dishes by trypsinization, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin (BSA; 1 mg/ml), and then seeded in 12-well plates that were either uncoated or precoated with fibronectin (30  $\mu$ g/ml). After 60 min, the percentage of attached cells was measured by the direct counting of attached and unattached cells using a Coulter counter or by scintillation counting of radiolabeled cells as described by Grinnell and Feld (21). The percent attachment was calculated as  $100 \times [\text{attached}/(\text{attached} + \text{unattached})]$ .

**Mitogenic response.** Mitogenic response of T47D cells was performed as previously described (45). Briefly, cells were passaged at  $10^5$  cells/well (24-well plate) and grown either on plastic, laminin, fibronectin, collagen type I or IV, Matrigel, or on poly-L-lysine-coated plates and starved to arrest cell growth. A mitogenic response in the quiescent cells was induced with heregulin, fetal bovine serum or epithelial growth factor, and measured by [ $^3$ H]thymidine (6.7 Ci/Mm, 2  $\mu$ Ci/ml for 45 min) incorporation at different time points up to 24 h. After being labeled with [ $^3$ H]thymidine, cells were washed three times with 5% trichloroacetic acid at 0°C, dissolved with 0.1 M NaOH, and then radioactivity was measured by using a scintillation counter.

**RNA isolation and Northern blotting.** Total RNA was isolated using an RNA kit from QIAGEN. The electrophoresis and blotting were done as described (44). Briefly, equal amounts of RNA (~ 15  $\mu$ g) were denatured, separated by electrophoresis on agarose-formaldehyde gels, and transferred to a Hybond-N nylon membrane (Amersham-

Pharmacia Biotech). Membranes were hybridized with a 1179-bp fragment corresponding to cDNA sequences from 1025 to 2222 of BRCA1 (that was labeled with  $\alpha$ [ $^{32}\text{P}$ ]dCTP). Levels of mRNA were normalized to 18S rRNA or to actin after probing the stripped blots with labeled rat cDNA to 18S rRNA or to actin.  $^{32}\text{P}$  labeling of probes was carried out with a random primer DNA labeling kit from Boehringer-Mannheim (Indianapolis, IN). The cDNA fragment of BRCA1 was amplified by PCR as described by Gudas et al. (24), and the mouse 18S rRNA was kindly obtained from D.M. Templeton (University of Toronto, Ontario).

**MAPK activity.** MAPK activity was determined by the ability of the immunoprecipitated enzyme to phosphorylate myelin basic protein (MBP) (1) in an *in vitro* kinase assay. Immunoprecipitates were mixed with assay buffer containing 20 mM HEPES (pH 7.4), 10 mM  $\text{MgCl}_2$ , 2 mM  $\text{MnCl}_2$ , 0.5 mM EGTA, 10 mM NaF, 0.5 mM  $\text{Na}_3\text{VO}_4$ , 1 mM dithiothreitol, 0.5 mg/ml MBP, 100 mM ATP, and 5  $\mu\text{Ci}$  of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , and incubated at  $30^\circ\text{C}$  for 30 min. The reaction was stopped by the addition of sample buffer for the electrophoresis according to Laemmli (35), and the mixture was separated on 15% SDS-PAGE for the silver staining and autoradiography.

**PI3K activity.** Cells were lysed in buffer A containing 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1% NP-40, 1 mM PMSF and 0.1 mM  $\text{Na}_3\text{VO}_4$ , and then cytosolic extracts were immunoprecipitated with anti-phosphotyrosine antibody (PY20). Precipitates were subjected to an *in vitro* kinase assay using  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and phosphatidylinositol as substrates, according to Derman et al. (12). Briefly, beads were washed and incubated for 10 min at room temperature in kinase buffer containing 0.5 mM ATP, 20 mM  $\text{MgCl}_2$ , 50 mM HEPES, pH 7.0, 0.25 mg/ml phosphatidylinositol and

30  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP (3000 Ci/mmol). Lipids were then extracted by  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (1:1) mixture, separated on oxalate-coated thin-layer chromatography plates (EM Science, Gibbstown, NJ) in developing solution containing  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{NH}_4\text{OH}$  [60:47:11,3:2], followed by autoradiography.

**Immunoblotting.** Cells were lysed in lysis buffer A, and lysates were subjected to SDS-PAGE according to Laemmli (35). Separated proteins were transferred to PVDF membranes in 25 mM Tris and 192 mM Glycine (pH 8.3) containing 15% methanol, and then blocked with 5% BSA and 5% Carnation milk in 30 mM Tris-HCl (pH 7.4) containing 137 mM NaCl, 2.6 mM KCl and 0.05% Tween 20. Membranes were then probed with either anti-Erk-2 antibody, polyclonal rabbit anti-phospho-Erk antibody, monoclonal mouse anti-phosphotyrosine antibody 4G10, monoclonal mouse anti-actin antibody, polyclonal rabbit anti-Akt, polyclonal rabbit anti-phospho-Ser-473 Akt antibodies or polyclonal anti-ErbB-2 antibody, and immunoreactive bands were detected with the NEN-Biolab (Boston, MA) enhanced chemiluminescence system, followed by autoradiography.

## RESULTS

**Extracellular matrix (ECM) modulates heregulin (HRG)-dependent mitogenic response of T47D cells.** T47D breast cancer cells proliferate uniformly when treated with growth factors (33). To study the effect of substratum on the mitogenic response of T47D cells, the incorporation of [ $^3$ H]thymidine into DNA was measured. Cells grown on plastic (PL) showed a maximal DNA synthesis between 12 and 20 h, peaking at 18 h after treatment with heregulin (HRG, 209  $\pm$  22% vs. 100  $\pm$  38% of untreated cells; Fig. 1A). A similar response was obtained when T47D cells were grown on fibronectin (FN, 206  $\pm$  13%, at 18 h). Cells seeded on collagen type IV (COL4) strongly suppressed the mitogenic response (92  $\pm$  38%, at 18 h), whereas the peak of thymidine incorporation was markedly increased in those cells grown on laminin (LAM) or Matrigel (MTR) (276  $\pm$  10% and 264  $\pm$  11%, at 18 h, respectively). Cells seeded on poly-L-lysine (POL) showed no difference in DNA synthesis whether in the presence or absence of HRG (100  $\pm$  16% vs. 100  $\pm$  13%), suggesting the involvement of integrins, receptors for the extracellular matrix, in HRG-dependent mitogenic response. Indeed, T47D breast cancer cells were found to express  $\alpha_6$ ,  $\beta_1$  and  $\beta_4$  integrins, as indicated in Fig. 1e. T47D cells adhered similarly to all substrata used in this study [PL, LAM, FN, COL4 and POL, (data not shown)] suggesting that the differences in mitogenic response were not due to different number of cells attaching to different substratum. Thus, T47D cells proliferated to a comparable level when seeded on PL and FN, while demonstrating decreased growth when seeded on collagen type IV or on poly-L-lysine. Cells grown on LAM or on MTR demonstrated the highest mitogenic response upon treatment with HRG.

To determine if ECM would affect DNA synthesis elicited by other growth factors, we compared the mitogenic response of cells stimulated with epithelial growth factor (EGF, 10 ng/ml, Fig. 1c) or fetal bovine serum (FBS, 10%, Fig. 1d) to that elicited by HRG (Fig. 1b). A representative time point of thymidine incorporation (at its peak; 18 h) was compared to non-treated cells (basal level, taken as 100%). While cells seeded on LAM and MTR responded to HRG with enhanced DNA synthesis, cells seeded on PL and FN as well as those grown on MTR had the greatest response to EGF (367  $\pm$  11%, 290  $\pm$  13% and 262  $\pm$  4%, respectively vs. 100  $\pm$  20% of non-treated cells, Fig. 1c). Although cells grown on PL (237  $\pm$  4%), LAM (320  $\pm$  8%) or MTR (259  $\pm$  9%) had a considerably high thymidine incorporation, those cells grown on FN had the highest peak of DNA synthesis after treatment with FBS (586  $\pm$  8%). Interestingly, DNA synthesis was strongly suppressed on both POL (118  $\pm$  10% at 18 h) and COL4 (97  $\pm$  33% at 18 h) coated plates after treatment with EGF, while cells treated with FBS responded modestly when seeded on these two matrices (POL, 152  $\pm$  45%; COL4, 171  $\pm$  39%, at 18 h). This modest effect could be attributed to the mixture of growth factors and cytokines found in FBS. Thus, laminin and also laminin-containing Matrigel had the strongest effect in enhancing HRG-dependent mitogenic response in T47D breast cancer cells.

**ECM modulates the HRG-dependent phosphorylation of BRCA1.** BRCA1 can be phosphorylated in response to DNA damage and in a cell cycle-dependent manner. Previously, we reported that BRCA1 was phosphorylated upon HRG stimulation (2). To investigate whether ECM may modulate BRCA1 phosphorylation, cells were grown on plastic plates, on plates coated with either LAM or POL, or were maintained in

suspension. Cells were starved for 48 h and metabolically labeled with [ $^{32}$ P]H<sub>3</sub>PO<sub>4</sub>. The phosphorylation of BRCA1 was then determined by immunoprecipitation with anti-BRCA1 antibody, followed by separation on SDS-PAGE. Cells seeded on PL showed a transient phosphorylation of BRCA1 in response to HRG, peaking at 0.5 – 1 h and returning to the basal level thereafter (Fig. 2a), supporting our previous work (2). Therefore, at the 1 h time point, representing the peak of HRG-dependent phosphorylation, the effect of ECM components on this process was tested. While the amount of BRCA1 protein was constant throughout the experiment as indicated in the lower panels of Fig. 2, the level of phosphorylated BRCA1 upon HRG treatment was enhanced in cells seeded on LAM as compared to those seeded on PL (Fig. 2b). The increased BRCA1 phosphorylation on LAM was suppressed by blocking antibodies for  $\alpha_6$  integrin, while blocking anti- $\beta_1$  integrin antibody had a modest effect, as indicated in Fig. 2c. Cells seeded on COL4 or those maintained in suspension showed no or very low phosphorylation of BRCA1. Taken together, these results indicate that LAM enhanced the HRG-mediated phosphorylation of BRCA1. This phenomenon was mediated by  $\alpha_6$  integrin.

**Is HRG-dependent signaling enhanced in cells seeded on laminin?** HRG-dependent phosphorylation of BRCA1 is regulated through the PI3K/Akt pathway after recruitment of PI3K to the activated receptor (2, 13). To investigate further the HRG-dependent intracellular signaling, cells were seeded on plastic, on plates coated with FN, COL, or POL, or were maintained in suspension and then compared to those grown on LAM. HRG increased the activity of various tyrosine kinases, which peaked at 10 min, as determined by anti-phosphotyrosine antibody (not shown). Therefore, the effect of ECM

on HRG-dependent downstream signaling was tested at 10 min, the peak of tyrosine phosphorylation induced by HRG. Total cell lysates were immunoprecipitated with anti-ErbB-2 antibody, subjected to SDS-PAGE and probed with anti-phosphotyrosine antibodies (Fig. 3a). Cells seeded on PL showed a strong increase in ErbB2 tyrosine phosphorylation. This phosphorylation was 2-3-fold stronger in cells grown on LAM, thereby corroborating the previous observations that LAM supported the strongest phosphorylation of BRCA1 and mitogenic response (Fig. 2b and Fig. 1b). Cells seeded on FN or on COL4 showed stimulation of the ErbB-2 receptor, however, it was much lower than that seen in cells grown on PL. The lowest tyrosine phosphorylation was observed in cells seeded on POL or in cells maintained in suspension (Fig. 3a).

**Laminin increases HRG-dependent PI3 kinase activity but not MAPK activity.**

PI3K activity was measured by an *in vitro* kinase assay and by western blotting using anti-phospho-Ser<sup>473</sup> AKT antibodies. The highest PI3K activity was observed in cells grown on LAM, and the lowest was seen on FN and COL4 (Fig. 3b and 3c). Samples from untreated cells were not able to significantly phosphorylate phospholipids. To address the specificity, several kinase inhibitors were used prior to the addition of HRG. As expected, LY294002 totally inhibited HRG-dependent PI3K activity in these cells, while PD98059, a known MEK inhibitor, was not effective (Fig. 3b and 3c). Similar to our previous study (2), MAPK did not play a significant role in this process as determined by western blotting using anti-phospho-MAPK antibodies and by an *in vitro* kinase assay (Fig. 4a and 4b, respectively). The strongest MAPK activity was observed in cells grown on PL or FN, while cells grown on COL4 or LAM, or those maintained in suspension showed modest MAPK activity after treatment with HRG (Fig. 4a and 4b).



Therefore, the highest BRCA1 phosphorylation as seen in cells grown on LAM (Fig. 2b) also correlated with greater intracellular signaling on the same substratum (Fig. 3).

**Effect of ECM on BRCA1 expression.** Because ECM affected the phosphorylation of BRCA1 in the presence of growth factors, we analyzed whether ECM also affects the expression of BRCA1. Over an extended time course, we observed a biphasic effect of HRG on BRCA1 mRNA levels in T47D cells grown on plastic. Specifically, we noted a high basal level of BRCA1 mRNA in non-treated cells which decreased rapidly up to 4 h after HRG treatment. This was followed by an increase in BRCA1 mRNA, peaking at 12 – 16 h, and by a decrease thereafter which approached the basal level (not shown). Based on these initial observations, we decided to compare the effect of ECM components on the expression of BRCA1 in non-treated or HRG-treated cells. The five most characteristic times were taken as study points including 0, 4, 14, 24, and 48 h after treatment with HRG and then compared to those time points after treatment with DMSO (Fig. 5a, 5b, and 5c). A characteristic biphasic pattern of BRCA1 expression after the treatment with HRG was observed in cells grown on plastic, laminin or collagen type IV. However, there was lower expression of BRCA1 in cells grown on laminin and the lowest expression in cells grown on collagen type IV (Fig. 5a, 5b, and 5c). This decrease of BRCA1 mRNA in T47D cells after growth on either laminin or collagen type IV was not observed in HCC-1937 breast cancer cells (Fig. 5d). This result suggests that this phenomenon was mediated through the C-terminus of the BRCA1 molecule, as HCC-1937 cells express C-terminus truncated BRCA1 (8). In order to test whether this strong decrease in BRCA1 mRNA expression upon HRG treatment is dependent on protein synthesis or the stability of the mRNA, cells were treated with actinomycin-D or with

cycloheximide. There was no difference in BRCA1 mRNA pattern when cells were treated with HRG alone (Fig. 6a) or HRG together with actinomycin-D (Fig. 6b). However, treatment of cells with cycloheximide (Fig. 6c) reversed the effect of HRG, as the mRNA expression of BRCA1 was increased 2 and 4 h after addition of the growth factor. This suggests the dependence of this phenomenon on protein synthesis. To further elucidate the decrease in BRCA1 mRNA level 4 h after treatment with HRG, cells were also treated with ALLN (a calpain inhibitor) or with lactacystin (a proteasome inhibitor) prior to the addition of HRG. As indicated in Fig. 6d, the HRG-dependent decrease in the BRCA1 mRNA level was not affected by pretreatment of cells with ALLN or lactacystin alone, while both inhibitors combined overcame the effect of HRG. In addition, another proteasome inhibitor, PSI, had a modest effect on the HRG-dependent BRCA1 mRNA level when compared to that of ALLN and lactacystin together (not shown).

**Effect of ECM and HRG on BRCA1 protein levels in T47D cells.** The strong basal level (Fig. 7a) of BRCA1 protein was decreased 4 h after treatment with HRG (Fig. 7b and 8A). Treatment of cells with DMSO, a solvent for HRG, did not decrease the level of BRCA1 protein (Fig. 7a and 7b). Cells were also seeded onto collagen type IV and laminin to test whether these matrices would affect protein expression as they did the BRCA1 mRNA. Doublet bands of BRCA1 protein were detected in the total lysates from cells seeded on both laminin and collagen type IV, which could be attributed to alternative splicing of the BRCA1 protein. Cells grown on collagen IV or on laminin had comparable levels of BRCA1 protein in non-treated cells (Fig. 7c and 7d). Similar to the cells adhering to plastic, there was a decrease in BRCA1 protein level 4h after

stimulation with HRG, and this level further decreased up to 24 h (Fig. 7c and 7d). The protein loading did not affect the results as the membranes were stripped and re-probed with either anti-actin antibody or with anti- ErbB2 antibody (Fig. 7a, 7b, 7c and 7d, lower panels). Similar to the expression of mRNA, the low BRCA1 protein level 4 h after treatment with HRG was restored after pre treatment with the proteasome inhibitor, PSI, suggesting the involvement of proteasome in this process (Fig. 8a). Four hour exposure of T47D cells to cycloheximide or to actinomycin-D, together with HRG, down-regulated BRCA1 protein to undetectable levels, a similar effect as when cells were treated with HRG alone. The results also suggest the involvement of both processes, protein synthesis and the stability of mRNA, in the suppressive effect of HRG on BRCA1 protein expression in these cells.

## DISCUSSION

An altered interaction between cells and surrounding ECM is a common feature of a variety of tumors (74). In breast cancer, epithelial cells are either incapable of producing an organized basement membrane (BM), which would normally induce growth arrest, or malignant cells fail to recognize their ECM because of expression of inappropriate or non-functional integrins (74). Conversely, it is possible to restore normal cellular differentiated function by correcting cell-ECM interactions (10, 28, 65, 74). Furthermore, integrins, including  $\alpha_6\beta_4$ , can regulate intracellular signaling cascades such as MAPK and PI3K (61, 75). Given that the level of BRCA1 is significantly lower in invasive breast cancer as compared with normal breast epithelial tissue (69), and that BRCA1 is phosphorylated in an HRG-dependent manner via the PI3K pathway (2), we postulated that ECM could affect the expression and phosphorylation of this tumor suppressor gene. Therefore, the effects of basement membrane components, such as laminin and collagen type IV, on BRCA1 expression and phosphorylation were studied. We observed that both HRG and ECM affected the expression and phosphorylation of BRCA1. HRG-dependent proliferation of T47D cells was highest in cells seeded on laminin or on laminin containing Matrigel, while the greatest mitogenic response to EGF or to FBS was seen in cells seeded on plastic or on fibronectin, respectively. The phosphorylation of BRCA1, which was mediated through  $\alpha_6$  integrin, was highest in cells seeded on laminin when compared with cells seeded on plastic or poly-L-lysine or to those maintained in suspension. Likewise, HRG-dependent intracellular signaling which leads to BRCA1 phosphorylation, such as through the PI3K/Akt pathway, was also enhanced in cells grown on laminin. We also found strong suppressive effects of both

laminin and collagen type IV on the mRNA level of BRCA1, which were mediated through its C-terminal. Treatment with HRG caused a biphasic response of BRCA1 mRNA expression in cells seeded on all three substrata (plastic, laminin and collagen type-IV); an initial decrease peaking 4 h after treatment, followed by a strong increase peaking at 14 h. The strong decrease in BRCA1 expression 4 h after treatment with HRG was mediated through protein degradation.

Stimulation of T47D breast cancer cells with heregulin induced cell cycle progression with the peak of DNA synthesis between 16 - 20 h. In a similar, proliferation-linked fashion, BRCA1 mRNA expression was observed on all three matrices tested in the present work. This pattern of BRCA1 expression resembled those reported earlier in T47D cells (25), MCF-7 cells (25, 71) and in normal epithelial cells (23, 42, 49, 51, 71). The significantly lower BRCA1 mRNA level observed in cells grown on laminin or on collagen type IV suggests that basement membrane components possess a mechanism that down-regulates BRCA1 mRNA. This response could be induced through integrins expressed in T47D cells. Indeed, T47D cell proliferation was blocked in cells grown on poly-L-lysine (Fig. 1) and BRCA1 phosphorylation was suppressed by blocking anti- $\alpha_6$ -integrin antibodies (Fig. 2c). The strong effect of collagen type IV on BRCA1 mRNA levels implicates  $\beta_1$ ,  $\alpha_1$  and  $\alpha_2$  integrins (collagen receptors) in addition to  $\alpha_6\beta_4$  (laminin receptor). Recently, it was implied that Matrigel matrix suppressed expression of BRCA1 in normal breast epithelial cells (49). These authors attributed this suppressive effect of Matrigel to laminin. However, in our study, we found that collagen type IV was clearly the most potent in suppressing BRCA1 mRNA expression. Thus, it will be of interest to

examine whether heparan sulfate proteoglycans, entactin, nidogen and TGF- $\beta$ , as components of Matrigel matrix (32), may participate in such effects.

ECM-dependent BRCA1 mRNA down-regulation was mediated through the C-terminus of the BRCA1 molecule. The level of BRCA1 mRNA was not significantly affected by the plating of HCC-1937 breast cancer cells on either laminin- or collagen type IV-coated plates. These cells contain C-terminus truncated-BRCA1 protein due to a gene mutation after codon 1755 (70). This region encompasses one and touches the second BRCT domain of the BRCA1 molecule, thereby disrupting their function (54). The BRCT domain is defined by a distinct cluster of 95 amino acids and is involved in protein-protein interactions (7). Recently, it was shown that activated SMAD2 bound to the Swift protein through the BRCT domain and elicited TGF- $\beta$ 1-induced gene regulation (62). Therefore, it is possible that the TGF- $\beta$ 1-dependent down-regulation of BRCA1 (57) in cells containing wild type BRCA1 is absent in HCC-1937 breast cancer cells, as the BRCA1 in these cell lacks the BRCT domain necessary for binding activated SMAD2.

The strong decrease in BRCA1 protein and mRNA expression 4 h after stimulation with HRG suggests that this time point may be important in the regulation of BRCA1 expression, because it occurred just before G1/S boundary elevation of the BRCA1 mRNA. Treatment of cells with general inhibitors such as actinomycin-D and cycloheximide (Fig. 7) suggests that this phenomenon was dependent on protein synthesis. Experiments with more specific inhibitors such as ALLN and/or lactacystin implicated the requirement of both calpain and proteasome in this process. Interestingly, neither ALLN nor lactacystin alone had a significant effect on BRCA1 mRNA

expression. This result is similar to those of Blagosklonny et al. (6) who reported that ALLN treatment did not affect BRCA1 mRNA levels in the cell lines they had tested. Both proteases, calpain and proteasome, could be activated under our experimental conditions. On the one hand, calpain is activated in response to  $[Ca^{2+}]$  elevation (77) initiated by integrin-ECM interactions (16, 52). In this regard,  $\beta_1$  integrin was shown to be involved in initiating  $[Ca^{2+}]$ -dependent pathways (63). On the other hand, proteasome could be activated by the phosphorylation of target proteins such as that achieved after stimulation with HRG in the present work. Thus, it is possible that cell attachment on laminin caused activation of proteasome through the phosphorylation of BRCA1, while attachment on collagen type IV activated calpain through  $\beta_1$  integrin, each causing a decrease in BRCA1 mRNA expression.

A significant reversal of the HRG-dependent decrease in BRCA1 protein levels 4 h after treatment with PSI or lactacystin suggests the involvement of proteasome-dependent protein degradation. The BRCA1 molecule contains a RING finger motif at its N-terminus (43, 54) which was linked to the activation of ubiquitin ligase activity (30, 54). Recent reports demonstrated that BRCA1 promoted ubiquitin polymerization by itself (41) and in concert with another RING finger-containing protein, BARD1 (27). Ruffner et al. showed that mutations in the RING finger domain abolished the ubiquitin ligase activity of BRCA1, thereby linking this activity of BRCA1 to its  $\gamma$ -radiation protection function (54). Phosphorylated BRCA1 could become a target of ubiquitination and subsequent degradation, as a number of substrates are known to require phosphorylation prior to ubiquitination. For instance, I $\kappa$ B $\alpha$  as a target protein is recognized by the SCF complex after being phosphorylated at Ser<sup>32</sup> and Ser<sup>36</sup> (17). However, so far, there has

been no link shown between the ubiquitination of BRCA1 and its degradation. Recently, it was shown that p53, another tumor suppressor gene, was targeted, ubiquitinated and subsequently degraded upon activation of the Her/Neu signaling cascade (78). Similarly, as shown in our experiments, Zhou et al. reported that the PI3K/Akt signaling pathway was activated, thereby producing MDM2 phosphorylation, nuclear localization and association with CBP/p300. As CBP/p300 also interacts with BRCA1 (50), it is possible that CBP/p300 provides a platform for the assembly of the protein complex which is necessary for the MDM2-mediated ubiquitination and degradation of BRCA1 (22). Our results corroborate those of Blagosklonny et al. who reported that BRCA1 levels are regulated by protein degradation in cancer cell lines with a low steady state of BRCA1 protein (6). They indicated specifically that cathepsin-like protease degradation of BRCA1 was in balance with BRCA1 transcription. However, our results differ from those of Aprelikova et al. who reported no change in BRCA1 protein level in starved MCF-10A cells or in MCF-10A cells stimulated with EGF or NDF (4). This difference could be attributed to the use of different techniques or cell lines.

The data reported here are consistent with the idea that growth factors and integrins can synergize to mediate biological processes, and that such collaborative action requires integrin aggregation and receptor occupancy (46, 67, 72). Similarly, the increased phosphorylation of BRCA1 on laminin was mediated through  $\alpha_6$  integrin while in the same cells, ErbB2 along with PI3K showed enhanced activity. Our results support the findings of Falcioni et al. who showed that the increased DNA synthesis observed in carcinoma cells treated with  $\alpha_6$ -integrin ligand was mediated through elevated ErbB2 phosphorylation (14).  $\beta_4$  integrin is most likely also involved in this process as



Gambaletta et al. reported that expression of both  $\alpha_6\beta_4$  integrin and ErbB2 was required for activation of PI3K. The authors further identified a specific region within the cytoplasmic domain of  $\beta_4$  that was essential for cell invasion (18). The laminin receptors,  $\alpha_6\beta_4$  and  $\alpha_6\beta_1$  integrins, but not  $\alpha_5\beta_1$ ,  $\alpha_3\beta_1$  and  $\alpha_2\beta_1$  integrins, associate with and activate ErbB2 receptors as demonstrated by coprecipitation of cell lysates from human carcinoma cell lines (14). This association was shown to be meaningful as  $\alpha_6\beta_4$  integrins together with ErbB2 were able to activate PI3K and thereby promote cell invasion (18) and proliferation (14). Along with the synergism between growth factors and integrins, it seems that different growth factors cooperate with different sets of integrins in order to regulate oncogenic pathways. For instance, in the present work, while the strongest proliferation in response to heregulin was observed in cells grown on laminin, the maximal mitogenic response upon EGF or FBS treatment was achieved in cells seeded on plastic or fibronectin, respectively. While the strong inhibitory effect of poly-L-lysine on cell proliferation indicates integrin involvement in this process, the effect of collagen type IV is somewhat surprising. Cells grown on collagen type IV had suppressed proliferation accompanied by decreased BRCA1 expression, and suppressed HRG-dependent phosphorylation, while cells seeded on laminin showed decreased BRCA1 expression, as well as increased HRG-dependent phosphorylation and cell proliferation. One possibility is that cells adhering to collagen type IV enhanced the expression and activation of  $\beta_{1C}$  integrin, which in turn could inhibit the proliferation of both tumorigenic and nontumorigenic cells by increasing expression of  $p27^{kip1}$  (15). It would be interesting to see whether these cells seeded on collagen type IV have enhanced expression of  $\beta_{1C}$  integrin and this CDK inhibitor.

BRCA1 can be phosphorylated in a cell cycle- (55) and DNA damage-dependent (58, 68) manner. Phosphorylation of BRCA1, as we indicated earlier, could also occur at the region spanning the nuclear translocation sequence after cell stimulation with heregulin. This phosphorylation was regulated through a PI3K/Akt-dependent pathway (2), however, the kinase responsible is still at large. One possibility is that the phosphorylation of BRCA1 is elicited by CDKs, as BRCA1 possesses four CDK consensus sites and was shown to be phosphorylated by CDK2 (53). Likewise, because it peaks between 0.5 and 2 h, HRG-dependent phosphorylation may be mediated through another CDK that is active earlier in the cell cycle, such as CDK4 or CDK6. Indeed, HRG activates both CDK6 (36) and CDK4 (38) and BRCA1 interacts with cyclin-D (73), suggesting that BRCA1 could be phosphorylated with a cyclin-D associated kinase. Although Ruffner et al. demonstrated that, in an *in vitro* assay, CDK6 could weakly phosphorylate BRCA1 protein, CDK6 involvement in this process seems unlikely. Only CDK4, and not CDK6, could be co-immunoprecipitated with BRCA1 interacting proteins (73). Based on our experiments, HRG-dependent CDK4 activity peaked 2 – 3 h after stimulation (unpublished observation; T. Miralem and H. Avraham). Thus, it is possible that this kinase phosphorylates BRCA1 just before it reaches its peak of activity. Studies are under way to address the kinase responsible for HRG-dependent BRCA1 phosphorylation and the specific phosphorylated sites.

In summary, we showed that both HRG and ECM could affect the expression and phosphorylation of BRCA1. While HRG induced BRCA1 phosphorylation through a yet unidentified kinase, it also induced a strong decrease in BRCA1 mRNA and protein level through protein degradation. Laminin enhanced HRG-dependent BRCA1

phosphorylation which resulted in increased cell proliferation, whereas both laminin and collagen type IV caused a C-terminus-dependent decrease in BRCA1 mRNA expression. However, collagen type IV suppressed the HRG-dependent mitogenic response in these cells. The linkage between HRG-dependent phosphorylation and the decreased BRCA1 expression on the one side and between BRCA1 and integrin expression on the other, remains to be determined in the future studies.

## FIGURE LEGENDS

FIG. 1. Effect of ECM on the HRG-dependent mitogenic response of T47D cells.

(a) T47D cells were seeded on either 24-well plastic plates ( PL; ●, ○ ) or on plastic wells coated with fibronectin (FN, ▲), laminin (LAM, □ ), matrigel (MTR, ■), collagen type IV (COL4, ◇ ) or poly-L-lysine (POL, △ ) and then starved for 48 h in medium containing 0.4% FBS. Cells were untreated (○ ) or treated with HRG (20 nM) for the indicated times and the medium was replaced with one that contained [<sup>3</sup>H]thymidine (2 μCi/ml). After 45 min, the “hot” medium was washed out, cells were lysed with NaOH and cell remnants were collected into scintillation tubes to measure incorporated radioactivity.

To compare the effect of HRG (a and b) with other growth factors, cells seeded as described in (a) were treated with EGF (10 ng/ml, (c), or with FBS (10%, d). The representative level of thymidine incorporation (at the peak of DNA synthesis) was compared to non-treated cells (taken as 100%). Values are expressed as the means (%) +/- s.d. of quadruplicate wells from three separate experiments. (e) Total cell lysates from T47D cells were processed for Western blotting. Membranes were probed with anti-β<sub>1</sub>, anti-β<sub>4</sub>, or anti-α<sub>6</sub> antibodies.

FIG. 2. ECM affects the HRG-dependent phosphorylation of BRCA1.

(a) Cells were seeded on plastic and starved for 48 h in growth medium containing 0.4% FBS. Four hours prior to stimulation with HRG (20 nM), cells were loaded with [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub>. After stimulation for the indicated times, total cell lysates were immunoprecipitated with anti-BRCA1 antibody, BRIN 129.5. Immunoprecipitates (IPs)

were subjected to SDS-PAGE and incorporated radioactivity was visualized by autoradiography (upper panel). IPs were also subjected to Western blotting and membranes were probed with BRIH monoclonal anti-BRCA1 antibody (lower panel).

(b) Cells seeded on plastic (PL), laminin (LAM), poly-L-lysine (POL) or maintained in suspension (SUS) were starved, metabolically labeled with [ $^{32}$ P], treated with HRG and lysed as indicated in Fig. 2A. Immunoprecipitates were processed the same way as indicated above for the upper and lower panels. (c) Cells grown on LAM coated plates or on PL were processed the same way as indicated above. Prior to stimulation with HRG, some plates coated with LAM were pre-treated with blocking anti- $\alpha_6$  or blocking anti- $\beta_1$  antibodies alone or together, and processed as indicated above. Autoradiographs are representative of two independent experiments.

FIG. 3. Laminin enhances the HRG-dependent activation of the ErbB2 receptor and PI3 kinase. (a) Starved T47D cells seeded on plastic (PL), laminin (LAM), collagen type IV (COL), fibronectin (FN), poly-L-lysine (POL) or those maintained in suspension (SUS) were stimulated with HRG for 10 min. Total cell lysates were immunoprecipitated with anti-ErbB2 monoclonal antibody and then immunoprecipitates were subjected to Western blotting. PVDF membranes were probed with 4G10 antibody (upper panel), or after stripping, with an anti-ErbB2 antibody (lower panel). "C" indicates a sample of total cell lysates, from stimulated cells grown on LAM, that was immunoprecipitated with protein-G-sepharose only. Some cells were treated with genestein (50  $\mu$ g/ml) for 15 min prior to the 10-min treatment with HRG at the peak of tyrosine phosphorylation. As a comparison to the treatment with HRG, some cells were stimulated with FBS. (b) Starved cells,

seeded on plastic (PL), laminin (LAM), collagen type IV (COL), or on fibronectin (FN) coated plates, were treated with heregulin (HRG, 20 nM) for 10 min. Total cell lysates were immunoprecipitated with either anti-phosphotyrosine antibody or with normal mouse IgG (IgG) for the *in vitro* kinase assay (b), or processed for Western blotting (c). (b) Immunoprecipitates were tested for their ability to phosphorylate lipids in the presence of [ $\gamma^{32}\text{P}$ ]ATP. The phosphorylated lipids were then extracted from the reaction mixture by  $\text{CHCl}_3:\text{CH}_3\text{OH}$  [1:1] and spotted on TCL plates. The extracted, phosphorylated lipids were then separated in developing solution followed by autoradiography. (c) Immunoblots were probed with anti-p-Ser473 AKT antibody (\*p-AKT, upper panel) or with anti-AKT antibody (lower panel) after stripping of the membrane. Some cells were pretreated with LY294002 [(10  $\mu\text{M}$ , (b) and (c)] or with PD98059 [(50  $\mu\text{M}$ , (b) and (c)]. The autoradiograms are representative of two separate experiments. "C" = reaction mixture without cell lysates. PIP = phosphatidylinositol phosphates.

FIG. 4. Laminin does not enhance the HRG-dependent activation of MAPK. T47D cells were seeded on plastic (PL), laminin (LAM), fibronectin (FN), collagen type IV (COL), or maintained in suspension (SUS). Starved cells were either left untreated or stimulated with heregulin (HRG, 20 nM) for 10 min. Obtained cytoplasmic cell lysates were either subjected to Western blotting (a) or immunoprecipitated with anti-Erk 1/2 antibody for the *in vitro* kinase assay (b). Some cells were treated with either PD98059 (50  $\mu\text{M}$ ) or with genestein (10  $\mu\text{M}$ ) prior to stimulation with HRG. (a) Blots of total cell lysates were probed with anti-phospho-Erk-2 antibodies (\*p-Erk, upper panel), and then stripped

and reprobed with anti-Erk-2 antibody as a control for total Erk protein (lower panel). C = sample buffer + RIPA buffer. (b) Immunoprecipitates were used for the *in vitro* kinase assay with [ $\gamma^{32}$ P]ATP and myelin basic protein (MBP) as a substrate. Control (C) indicates the kinase activity of the sample containing lysate derived from cells treated with HRG that was mixed with protein-G agarose with no anti-Erk-2 antibody. The lower panel represents immunoblots of immunoprecipitates probed with anti-Erk-2 antibody. Autoradiograms are representative of two independent experiments.

FIG. 5. Effect of HRG and/or ECM on BRCA1 mRNA expression in T47D and HCC-1937 cells. Cells were seeded on plastic (a) or on plates coated with laminin (b), or collagen type IV (c) and then starved for 48 h. Cells were next treated with HRG (20 nM, H) or with DMSO (D). At the indicated times, total RNA was collected using a Qiagen kit and 20  $\mu$ g of the total RNA was subjected to Northern blotting. (d) HCC-1937 and T47D cells were seeded on plastic (PL), laminin (L) or on collagen type IV (C) and starved for 48 h as indicated in Fig. 2. Total RNA was collected and processed the same way as described above. Blots in (a), (b), (c), and (d) were probed with an 1179-bp fragment of BRCA1 (upper panels) and with actin cDNA or 18S rRNA as indicated (lower panels). Results are representative of two independent experiments.

FIG. 6. Effect of various inhibitors on the level of BRCA1 mRNA in T47D breast cancer cells. T47D cells were seeded on plastic plates [(a), (b), (c) and (d)] and starved in growth medium containing 0.4% FBS. After 48 h, cells were either untreated (0) or treated with HRG alone (a) or with HRG in the presence of actinomycin-D (b) or cycloheximide (c) for the indicated times. (d) Some cells were also treated with ALLN

or lactacystin, alone or in combination, prior to the treatment with HRG for 4 h. Total RNA was extracted and processed the same way as indicated in Fig. 5. Autoradiograms are representative of two independent experiments.

FIG. 7. Effect of HRG, ECM and various inhibitors on BRCA1 protein level. T47D cells seeded on plastic [(a) and (b)], collagen type IV (c) or on laminin (d) coated plates were starved and treated with HRG (20 nM) [(b), (c) and (d)] or with DMSO [(a) and (b)] as a control for the indicated times (hours, h). (a) Total cell lysates were collected and subjected to Western blotting. Membranes were probed with anti-BRCA1 antibody (upper panel) or, after stripping, with anti-actin antibody (lower panel). (b) After cells were treated with HRG or with DMSO, total cell lysates were collected at the indicated times and subjected to Western blotting. Membranes were probed with anti-BRCA1 antibody (upper panel) or, after stripping, with anti-ErbB2 antibody (lower panel). [(c) and (d)] T47D cells were seeded on collagen type IV (20  $\mu$ g/ml, C) or on laminin (20  $\mu$ g/ml, d) coated plates, starved, and then stimulated with HRG for the indicated times. Total cell lysates were processed the same way as indicated in Fig. 7B. Membranes were stripped and re-probed with anti-actin antibodies. The blots are representative of three independent experiments.

FIG. 8. Effect of various inhibitors on BRCA1 protein level. (a) Cells seeded on laminin (L), collagen type IV (C) or on plastic (PL) were treated with HRG for the indicated times (H). Some cells grown on PL were pre-treated with PSI, before the addition of HRG. Total cell lysates were processed as described in Fig. 7A. (b) Starved T47D cells



seeded on plastic were treated with HRG in the presence of cycloheximide (CHX) for the indicated times and total cell lysates were analyzed. (c) Cells were treated with HRG and actinomycin-D (Act-D). The lower panels indicate the level of ErbB2 (a) or actin (b) and (c), after re-probing the membranes with the corresponding antibodies. The blots of all panels are representative of two independent experiments.

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Fig. 1

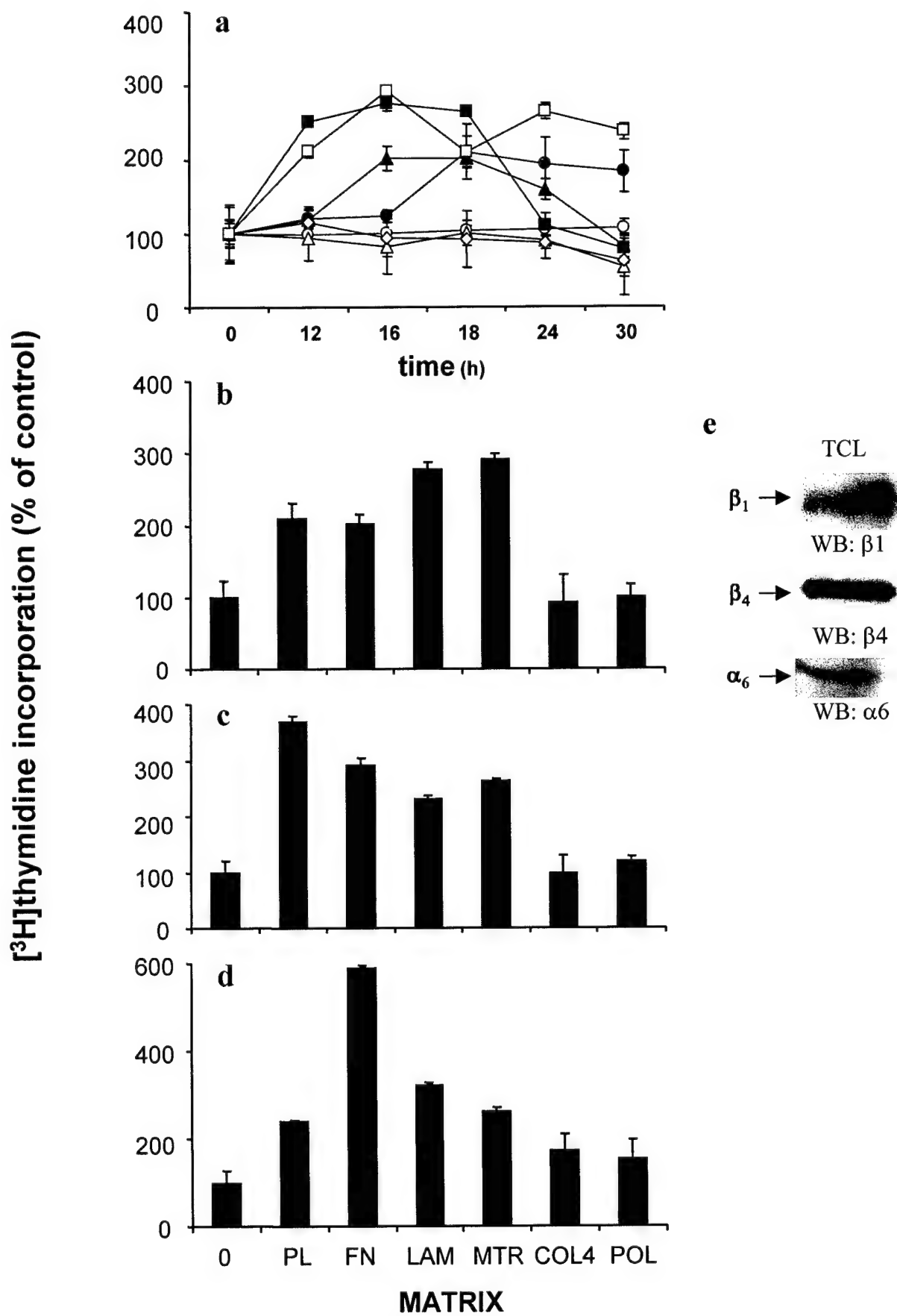


Fig. 2

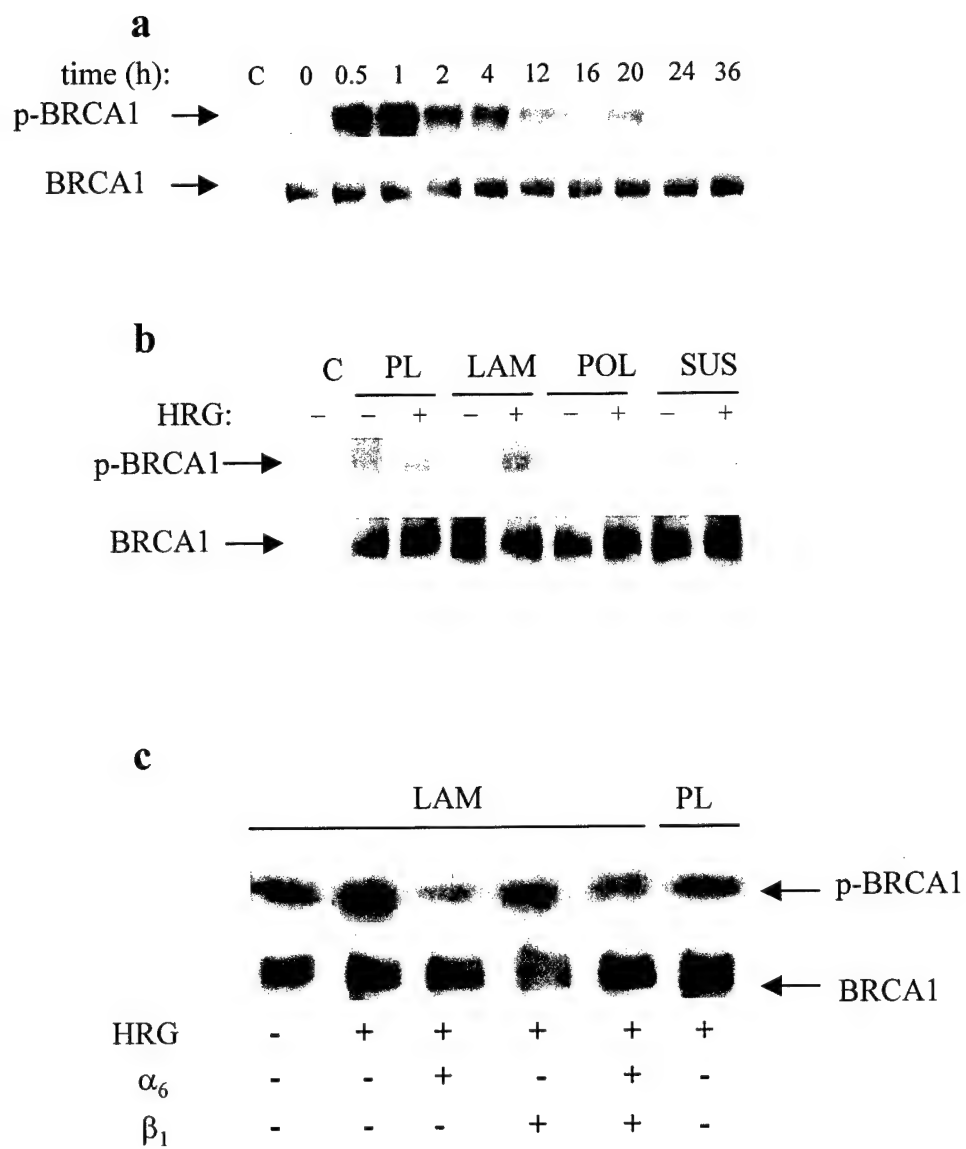


Fig. 3

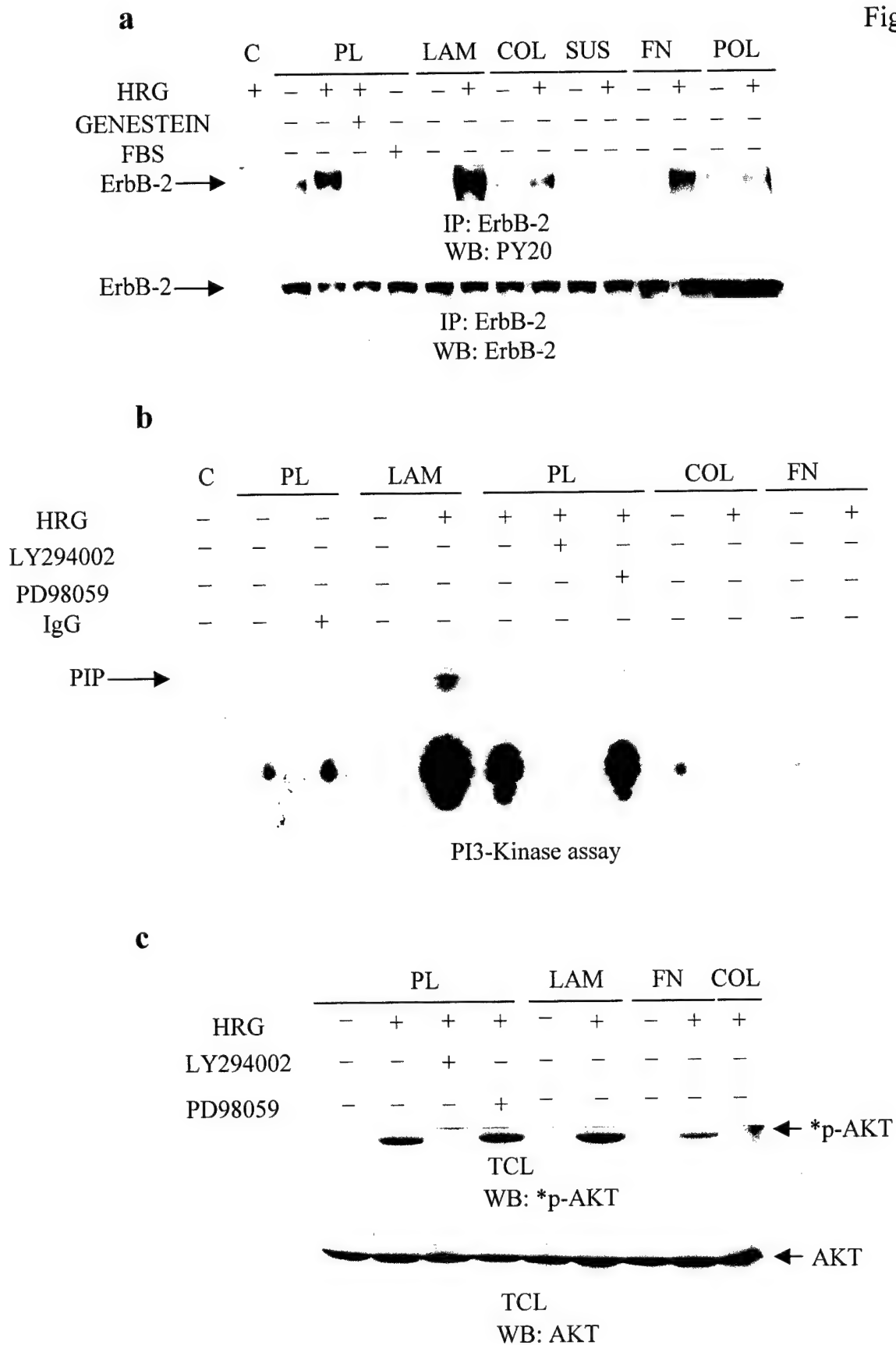


Fig. 4

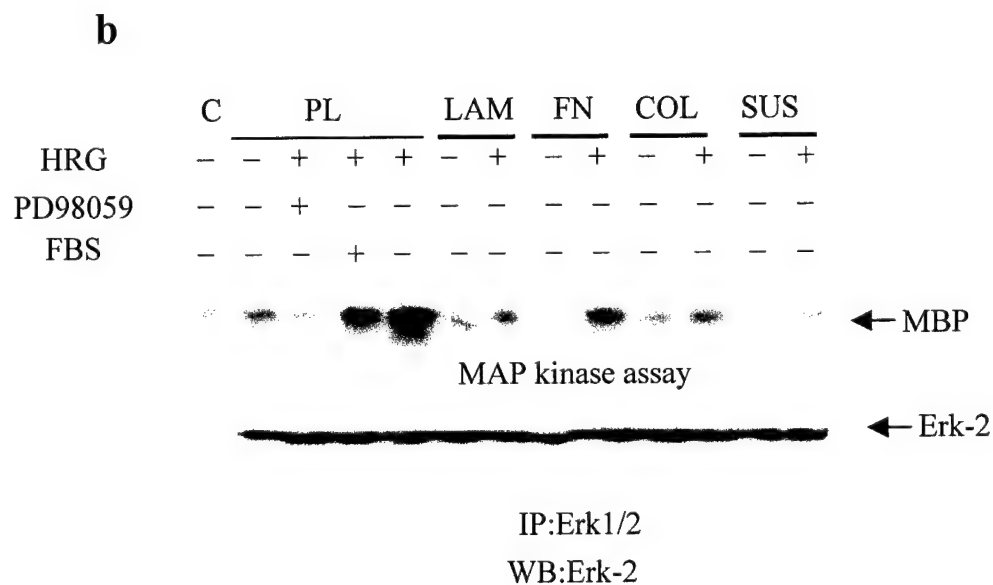
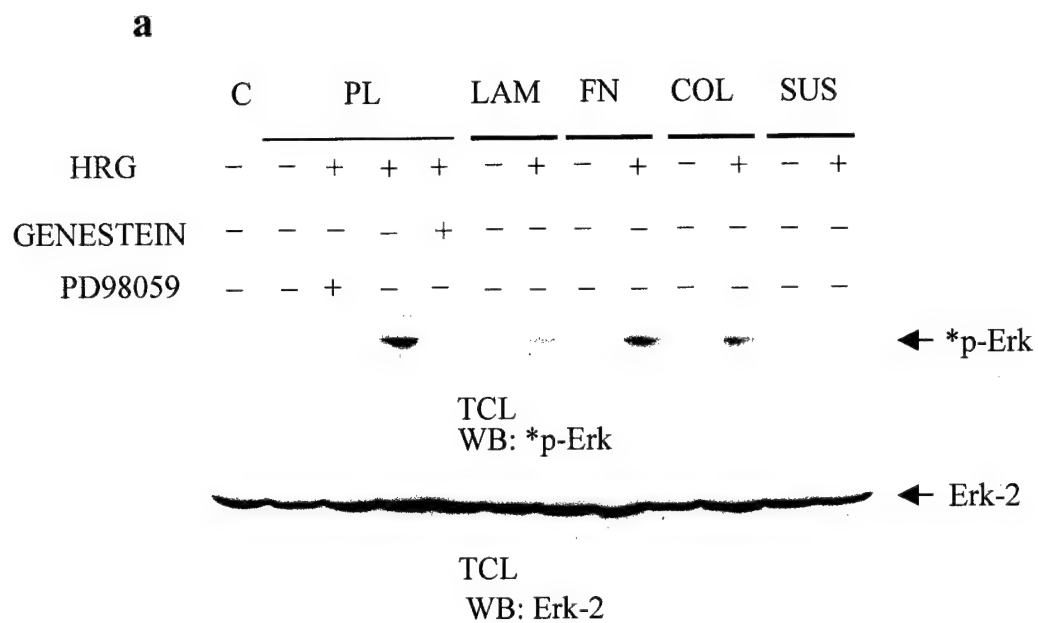


Fig. 5

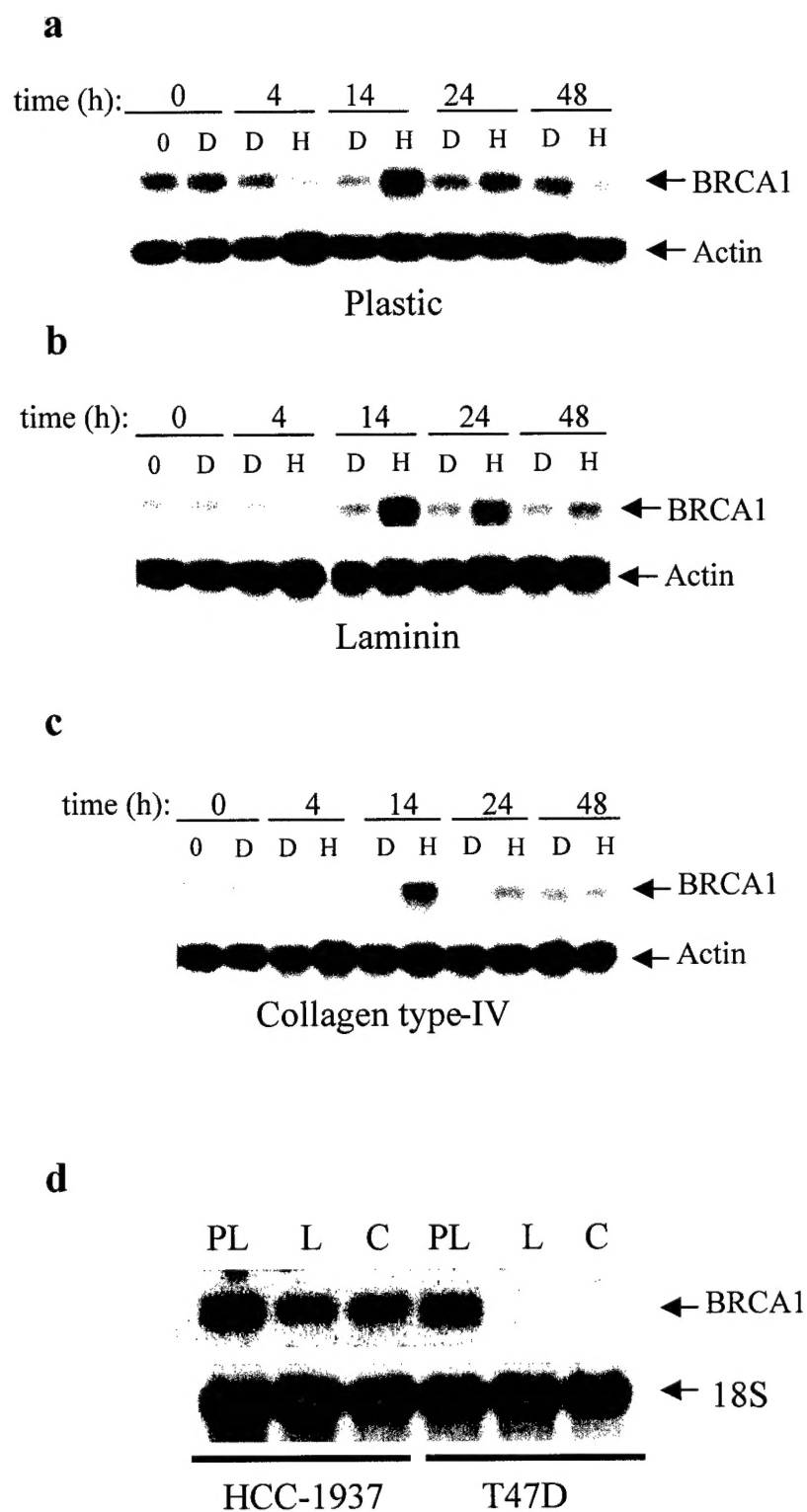
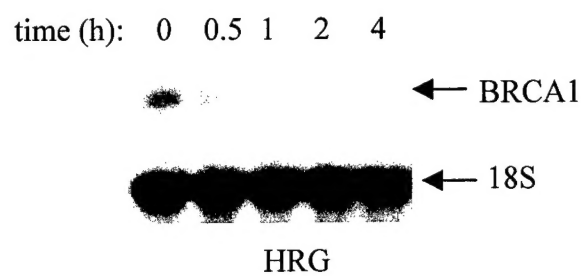
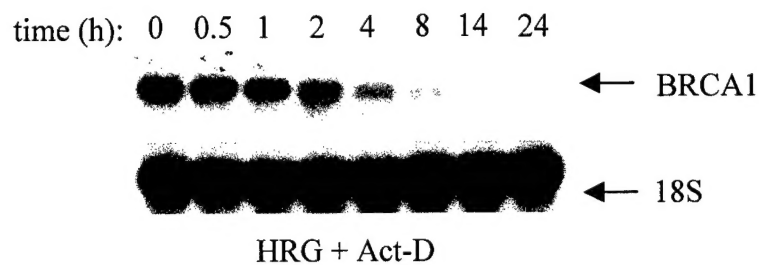


Fig. 6

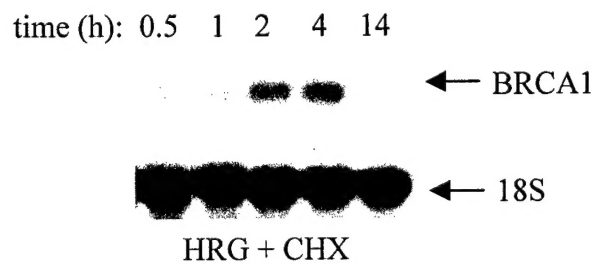
**a**



**b**



**c**



**d**

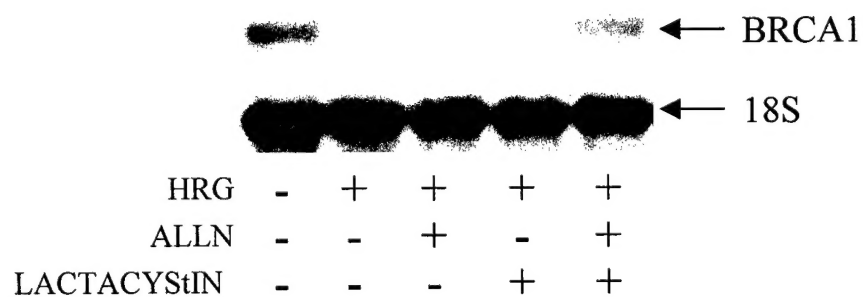


Fig. 7

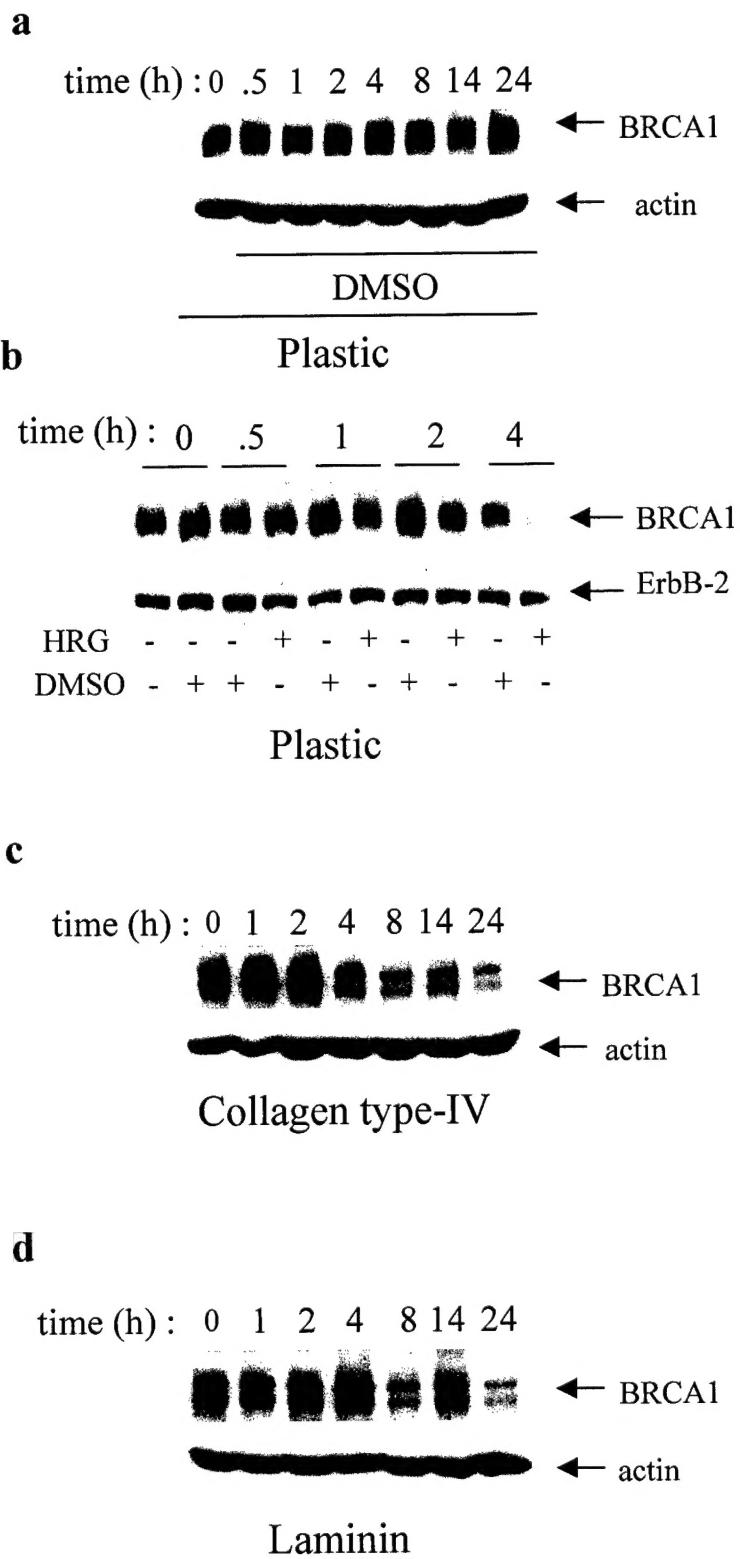


Fig. 8

